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THE
BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 12, 1966)

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio*, who shall be the Chairman, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti*, who shall be elected from present or former Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corration. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the officers. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of theTrustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEES' MEETINGS

EXECUTIVE COMMITTEE

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of not more than ten members including the *ex officio* members who shall be the Chairman of the Board of Trustees, President, Director and Treasurer; six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. (August 11, 1967).

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice President. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine. (August 12, 1966).

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or law. (August 16, 1963)

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its action shall be reported to the Board of Trustees. (August 16, 1963).

II. RESOLVED

The elected members of the Executive Committee shall be constituted as a standing "Committee for the Nomination of Officers," responsible for making nominations at the Annual Meeting of the Corporation and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Gentlemen:

In the important aspects of the life of the Marine Biological Laboratory this has been a good year. The scientific work has been maintained at a high level. Indeed, the activities of the scientists working at the Laboratory are a magnificent testimonial to the ability of good people to carry on good work regardless of financial stringency and budget cuts.

In terms of our physical plant, this has been the year in which the long-envisioned plans to house our instructional program in modern, fireproof quarters have been realized. As Conklin wrote in 1900, the wooden buildings all were of a temporary character and could be used only in summer. The then stated need for "at least one substantial, fire-proof building . . . which can be used the year around" increased with the years. Now three such year around laboratory buildings have replaced the wooden laboratory buildings to make possible the sustained service to scientists envisioned by Conklin. Although the passing of Old Main may be regretted by some, there can be no doubt that the Whitman and Loeb Buildings fulfill a long-standing need for contemporary structures. True, the quality of instruction depends in the first instance on the quality of the instructors and the students—of the senior and junior scientists, if you will. However, adequate modern fireproof quarters will certainly enhance the ability of our people to achieve the standards consistent with the changing requirements of modern science.

It is fitting here to recall the devotion with which Dr. Arthur Parpart as President of the Corporation of the Marine Biological Laboratory worked for the fulfillment of our building program. His untimely death in 1965 was a blow to us all; and the new buildings, completed and under construction, are a fine testament to the dedicated efforts of Arthur Parpart. We are fortunate indeed that the development of this program was carried out by Dr. Philip B. Armstrong initially during his tenure as Director and subsequently as Chairman of the Building Committee.

The MBL and the expanding world of marine sciences

The Marine Biological Laboratory was founded by a group of imaginative and dedicated scientists who visualized a facility serving the interests of all biology. Dr. Conklin, in a report written in 1900, speaks as follows:

"In his address at the opening of the Laboratory and in subsequent publications, Professor Whitman took the position that there was great need for a laboratory which should represent, (1) the whole of biology; (2) both teaching and research; (3) the widest possible cooperation of educational and scientific institutions. Such a laboratory should not be merely a collecting station, nor a summer school, nor a scientific work shop, nor a congress of biologists, but all of these; an institution combining in itself the functions and features of the best biological institutes of the world, having the cooperation of the biologists of this country, and thus forming 'a national center of instruction and research in every department of Biology.' The history of the Laboratory has shown that this ambitious project is not only highly desirable, but that it is entirely feasible and has justified the claim of the Director that such an institution is the greatest need of Americal biology."

The aims and aspirations set forth by C. O. Whitman and expounded by Conklin seventy years ago still have a scope and validity that is undeniable. The Marine Biological Laboratory has become indeed a national resource and all associated with the

Laboratory must share the responsibility of maintaining the viability of this intellectual asset of the world.

The Marine Biological Laboratory was founded by scientists with the major objective of getting on with science. The Laboratory is designed to provide services to biologists with facilitities open to all within the limits of physical space. The early evolution of the Laboratory depended not only on the efforts of scientists, but the growth for the first half century or more was also accomplished with the assistance of funds from individuals and private foundations. Now the pattern has changed. Federal funds are needed in larger proportion, and the research facilities of our sister institutions, the universities and colleges of the world, have expanded enormously over what they were many years ago. Clearly there is a continued and enhanced need to look to the future—to insure that the MBL continues to serve its fundamental purposes.

The new facilities, the Loeb Building and the new dining-dormitory complex, allow us to stretch our active calendar in a way that was not possible before. Particularly, the MBL can now truly cooperate with neighboring institutions of higher learning by making available superb seaside facilities for year around courses of instruction and advanced training. The fine effort by Boston University (Boston University Marine Program) has provided a valuable model for one such type of service we can render.

During the forthcoming season, plans will be developed to enhance the scope of activity of the MBL and to seek stable financing. We are indeed fortunate that Dr. James D. Ebert, Director-Designate, has assumed major responsibility for developing these forward-looking plans.

During the 1969 season, Dr. Case undertook the direction of the zoology course (Experimental Invertebrate Zoology), and Dr. Siegelman, the botany course (Experimental Botany). Both courses, under the new directors, achieved exemplary levels of innovation. Also, Dr. Adelman managed a most successful training program in Excitable Membrane Physiology and Biophysics. During the 1969 season, plans were made by Drs. Bennett and Dowling for the new course in neurobiology, and that program is demonstrating the imaginative approaches which we cherish at the MBL.

It is, of course, impossible to make any proper institutional assessment of the scientific advances made by independent investigators during their stay at MBL. The benefits of work here include not only research done in residence, but also ideas exchanged and new viewpoints generated. What can be said with great assurance is that the level of intellectual activity was clearly visible and was good.

A fine illustration of the potentialities of the MBL for extended season work occurred during this past year. Dr. Summers, in charge of our squid survey program, was able to provide squid to Dr. Adelman well on into the late fall. The squid were taken as a part of the ecological survey, and proved to be fine, healthy animals. It is now definitely established that squid users could be provided with animals throughout the year except during a few of the worse winter months. Thus squid join the ranks of other familiar organisms which are available to investigators on a year around basis.

The Systematics-Ecology Program

This program has celebrated its eighth anniversary. Well over a hundred major publications have appeared from work within or sponsored by the Program. Of special interest to the MBL in general has been the "Keys to Marine Invertebrates," of which nearly five thousand copies have been distributed through the Supply Department.

During the eight years of growth, 33 visiting investigators have been on deck, with 24 postdoctoral fellows and 16 graduate trainees. Field sampling for the biotic census of Cape Cod Bay has been completed, and the analysis of the results is proceeding. Staff members have contributed assistance to the Boston University Marine Program and to other visiting groups. The record of accomplishment of this innovative venture is very refreshing indeed and the program may well serve as one model for future year around development of our activities.

The future of the MBL is bright. The problems looming ahead are challenges, not obstacles. We welcome Dr. James Ebert as incoming Director and wish him well.

1. Memorial

ALBERT TYLER

By Charles B. Metz

Albert Tyler's sudden and untimely death on November 9, 1968 ended a distinguished career in developmental biology and a long association with the Marine Biological Laboratory. Albert Tyler was born in Brooklyn, New York on June 26, 1906. He began his higher education as a chemistry major at Columbia University, where he soon came under the influence of T. H. Morgan. Upon completion of the bachelor's degree in 1927, he became Morgan's graduate student. He obtained the master's degree at Columbia in 1928 and then moved to the California Institute of Technology with Morgan where he received a combined biology and chemistry Ph.D. in 1929. He was then appointed to the Institute's faculty and rose through the ranks to the professorship of embryology which he held the rest of his life. Tyler and Morgan were close friends and scientific colleagues for the remainder of Morgan's life. Both men were highly imaginative, novel and wide-ranging in their thinking and interests. Their special concerns were problems of developmental and marine biology. This last interest almost certainly originated from Morgan's experiences as a student at the Annisquam Marine Laboratory the summer of 1886 and led to his long association with its immediate successor, the Marine Biological Laboratory. In due course Morgan transmitted his interest in marine organisms and the Marine Biological Laboratory to Albert Tyler.

Albert Tyler's direct association with the Marine Biological Laboratory began the summer of 1926, following his junior year at Columbia. He was then listed as a Beginning Investigator at the Laboratory, a status he held each summer until 1929 when he was promoted to Independent Investigator. He was elected to Corporation Membership in 1931, served as an Instructor in the Embryology Course from 1946 through 1950, a Trustee of the Laboratory from 1948 to 1962 and a member of the Editorial Board of the Biological Bulletin from 1951 through 1954. His concern for the Laboratory and his influence on many of its activities were very great.

Albert Tyler was one of the leaders in the transition from classical experimental embryology to the molecular biology of development of the present decade. He was almost uniquely equipped for this role with his strong training in the physico-chemical sciences coupled with the very best possible instruction in classical genetics, cytology and developmental biology obtained from T. H. Morgan, E. B. Wilson and others at the time when these subjects were at their peak. Albert Tyler's first research was performed here at the Marine Biological Laboratory and followed the classical tradition. It concerned the production of double embryos in annelids and molluscs. Two papers and his Ph.D. thesis resulted from this work. His first officially recorded seminar at the Marine Biological Laboratory was delivered in 1931 on the subject of artificial parthenogenesis in the eggs of the Pacific Coast echiuroid, *Urechis caupo*. Shortly, however, his interests turned to more dynamic aspects of development. This was reinforced during the year 1932–33 while a National Research Council Postdoctoral Fellow in Warburg's Laboratory and at the Zoological Station at Naples. This new line of investigation concerned the energetics of development and particularly the energy requirements for form changes in the embryo. The first paper in this series appeared in 1933 and the work is now regarded as a classical application of physico-chemical principles to the study of developmental processes. This research led to studies on metabolic changes and particularly the increase in respiration that follows fertilization in sea urchin eggs. In the late 1930's Albert Tyler and several of his colleagues including S. H. Emerson, L. C. Pauling and A. H. Sturtevant recognized that the modern immuno-chemistry, then emerging, had significance for several biological disciplines and could also provide tools for study of other fundamental problems. Albert Tyler took this as an opportunity to explore the macro-molecular events of fertilization and development in a more modern and rigorous fashion. These studies and their offshoots held his interest for the next 20 years.

His investigations on fertilizin and antifertilizin modernized F. R. Lillie's antigen-antibody analogy, and extended our knowledge of fertilizin and antifertilizin chemistry, specificity, mechanism of interaction and role in fertilization. He investigated specific egg membrane lysins from spermatozoa of molluscs. These substances clearly can contribute to fertilization specificity and certainly are essential to provide a passage for the spermatozoan to the egg surface. In collaboration with Harry Grundfest and Alberto Monroy here at the Marine Biological Laboratory he demonstrated a resting potential and a change in potential at fertilization in the echinoderm egg. Later, electron microscope studies on sperm-egg interaction resulted in provocative theories to relate the ultrastructural observations to the role of fertilizin and antifertilizin in fertilization.

Characteristically, he introduced many conceptual and experimental novelties into his work. For example, to study the role of antigens in fertilization he developed a method for preparing non-agglutinating and non-precipitating antibodies by photo-oxidation some fourteen years before the discovery of enzymatic digestion methods for preparing univalent antibodies. His studies on sea urchins led him to formulate an autoantibody theory of cell structure which he proceeded to test by examining for specific complementary substances in pneumococci and for antivenom production in Gila monsters. Later he published a theory of cancer and, as usual, undertook extensive laboratory tests, in this case with mice. But problems of fertilization and reproductive physiology commanded his major interest during this period. His precise thinking and laboratory skill set the standard for all who worked in this field.

During the last ten years Albert Tyler concentrated on the molecular biology of fertilization and development. He was among those who first provided evidence that messenger RNA must be present in an inactive or "masked" form in the unfertilized sea urchin egg and he early appreciated that such stored informational RNA might constitute the classical "formative substances" responsible for determination in the egg. This began an intensive study of the nucleic acids of the egg including cytoplasmic DNA and the mechanism of activation of protein synthesis at fertilization. These investigations were cut off when, at age 62, Albert Tyler was at the height of his extraordinarily productive career.

The impact of Albert Tyler's published contributions will long endure. Likewise his influence will continue to be significant through the many students, postdoctorals and friends who received training and inspiration from him in his own laboratory, here at the Marine Biological Laboratory, and at other institutions during visits. Even brief visits had a lasting effect on students. This resulted from an extraordinary combination of energy, personality, imagination and immediate transfer of ideas to laboratory tests.

Albert is survived by his wife, Betty, and two sons: James, a successful engineer, and Steven, a graduate student at Santa Barbara. The family ties were very strong, and all shared Albert's enthusiasm for science and participated in it. Betty and James are co-authors on papers with Albert.

Albert Tyler was sought after to organize and chair many symposia and to function in advisory and consultant capacities to government agencies and private foundations. He served as President of the American Society of Naturalists and the Society of General Physiologists. Nevertheless, Albert was alway an informal man who abhored pretense and display. He especially enjoyed small informal gatherings. Here he shared his zest for science through discussion with friends. His many friends here at Woods Hole, as elsewhere, will long remember such discussions and miss the excitement and challenge of Albert's subtle wit and penetrating mind.

2. The Staff

EMBRYOLOGY

I. Consultant

Everett Anderson, Professor of Biology, University of Massachusetts

II. Instructors

Malcolm S. Steinberg, Professor of Biology, Princeton University, in charge of course
John M. Arnold, Assistant Professor of Cytology, Pacific Biomedical Research Center, University of Hawaii
Max Burger, Associate Professor of Biology, Princeton University
Gary Freeman, Assistant Professor of Biology, University of California at San Diego
Ralph T. Hinegardner, Associate Professor of Biology, University of California at Santa Cruz
Antone Jacobson, Professor of Biology, University of Texas
Hans Laufer, Associate Professor of Zoology, University of Connecticut

III. Lecturers

Lionel Jaffe, Professor of Biology, Purdue University
Raymond Rappaport, Professor of Biology, Union College
Paul B. Weisz, Professor of Biology, Brown University

IV. Laboratory Assistants

Norman A. Granholm, University of Oregon
Robert S. Turner, University of Oregon

V. Lectures

M. S. Steinberg	Introduction to the course
Antone Jacobson	Introduction to the embryology of teleost fishes
W. W. Ballard	Gastrulation in teleosts
Anna Ruth Brummett	Experiments on the dynamics of embryo formation in teleosts
Antone Jacobson	Experiments on the control of organ determination
M. S. Steinberg	Self-assembly of multicellular complexes
Herbert M. Phillips	Equilibrium measurements of embryonic cell adhesiveness
M. S. Steinberg	Morphogenetic phenomena in sponges
M. S. Steinberg	Developmental control processes in coelenterate ontogeny
Stanley Shostak	Form-determining mechanisms in hydra

Everett Anderson	The fine structure of eggs
Jean C. Dan	Fertilization
Ralph T. Hinegardner	Echinoderm development: egg to pluteus
Raymond Rappaport	Cytokinesis: establishment of the mechanism
Raymond Rappaport	Cytokinesis: nature and operation of the mechanism
Ralph T. Hinegardner	Echinoderms: life cycle and experimental embryology
Lewis Tilney	Microtubules in mesenchyme formation and differentiation in *Arbacia*
Paul Gross	Regulation of macromolecule synthesis I
Paul Gross	Regulation of macromolecule synthesis II
A. B. Pardee	Division of bacterial cells
Lionel Jaffe	Localization in the developing fucus egg, and the general role of localizing currents
Eric Davidson	Localization of "morphogenetic factors" in the cytoplasm of animal eggs, and its possible significance
John M. Arnold	Normal development of the spiralian embryo
John M. Arnold	An analysis of spiralian development
John M. Arnold	Development of the cephalopod embryo
Jack Collier	Gene transcription during *Ilyanassa* embryogenesis
Hans Laufer	Embryonic development in crustacea
Hans Laufer	Post embryonic development in crustacea
Herbert Oberlander	Effects of insect hormones on imaginal discs *in vitro*
Gary Freeman	The organization of the ascidian egg
Irwin Konigsberg	Clonal development of embryonic myoblasts *in vitro*
Gary Freeman	Metamorphosis and asexual reproduction in ascidians
J. Richard Whittaker	Pigment cell differentiation in ascidian embryo
Roger D. Milkman	Development and genetics of *Botryllus schlosseri*
Beatrice Mintz	Four-parent mice offer clues about gene control of mammalian differentiation
Paul B. Weisz	The significance of larvae

PHYSIOLOGY

I. Consultants

Merkel K. Jacobs, Professor of Physiology, University of Pennsylvania

Albert Szent-Györgyi, Director, The Institute for Muscle Research, Marine Biological Laboratory

W. D. McElroy, Director, McCollum-Pratt Institute, The Johns Hopkins University

J. Woodland Hastings, Professor of Biology, Harvard University

II. Instructors

Andrew G. Szent-Györgyi, Professor of Biology, Brandeis University, in charge of course

Sydney Brenner, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England

Roderick K. Clayton, Professor of Biophysics, Cornell University

Seymour S. Cohen, Professor of Biochemistry, University of Pennsylvania

Hugh E. Huxley, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England

Maurice Sussman, Professor of Biology, Brandeis University

David A. Yphantis, Professor of Biology, University of Connecticut

III. Special Lecturers

Harlyn Halvorson, Professor of Bacteriology, University of Wisconsin
Shinya Inoué, Professor of Biology, University of Pennsylvania
K. E. Van Holde, Professor of Physical Chemistry, University of Oregon

IV. Staff Associates

Raymond E. Stephens, Department of Biology, Brandeis University
Annemarie Weber, Department of Biochemistry, St. Louis University
Raquel Sussman, Department of Biology, Brandeis University
Peter Newell, Department of Biology, Brandeis University
Eugene Katz, Department of Biology, Brandeis University
Michael Johnson, Department of Biophysics, University of Connecticut
Dennis Roark, Department of Biophysics, University of Connecticut
Walter F. Stafford, III, Department of Biophysics, University of Connecticut
Peter B. Moore, Department of Molecular Biology, Yale University
Darrell Fleischman, Charles F. Kettering Research Laboratory
Celia E. Freda, Department of Therapeutic Research, University of Pennsylvania

V. Research Assistants

Richard Wayne Linck, Department of Biology, Brandeis University
Rita Burki, Department of Biochemistry, St. Louis University
Ellen Rayner, Department of Biology, Brandeis University
B. J. Clayton, Department of Genetics, Development and Physiology, Cornell University
Ellen Streibel, Department of Therapeutic Research, University of Pennsylvania

VI. Laboratory Assistants

Margaret Ketchum, College of Liberal Arts, Boston University
David Sloane Wilson, University of Rochester

VII. Lectures

Andrew G. Szent-Györgyi	Aspects of chemistry of muscle contraction
	Assembly and organization of the filaments of molluscan muscles
Annemarie Weber	Control of contraction and relaxation
Raymond E. Stephens	"All microtubules are equal but some are more equal than others"
David A. Yphantis	Physical approaches in biochemistry I
	Physical approaches in biochemistry II
	Physical approaches in biochemistry III
Roderick K. Clayton	Photosynthesis: ancient history: biochemical and evolutionary speculations
	Photosynthesis: breathes there a man with soul so tough, he thinks two systems aren't enough?
	Photosynthesis: the value of absorbtion and emission spectroscopy
Maurice Sussman	DNA and RNA in eucaryotic cells: an embarrassment of riches
	The cellular slime molds XLVI
	The cellular slime molds XLVII

Harlyn Halvorson	Developmental control in XLVIII
Seymour S. Cohen	Synthesis of phage proteins I
	Synthesis of phage proteins II
	Functions of polyamines
Harold S. Ginsberg	Synthesis of adenoviruses
Henry Mahler	Biogenetic autonomy of yeast mitochondria
Rachmiel Levine	Action of insulin
Hugh E. Huxley	X-ray diffraction results on striated muscle
	Functional implications of muscle structure
Ian R. Gibbons	Aspects of ciliary movement
Shinya Inoué	Inscrutable fibers of the spindle
Ruth Sager	What does chloroplast DNA do?
Bernard D. Davis	Ribosome polysome cycle
M. V. L. Bennett	Central dogma of neurophysiology I
	Central dogma of neurophysiology II
Lawrence B. Cohen	Changes in optical properties of nerves during activity
Edward A. Kravitz	Biochemical studies on single neurons and preliminary observations on lobster ganglia in long-term culture
Ruth Hubbard	Chemistry of visual photoreception
Richard Cone	Photoreceptor excitation mechanisms
John E. Dowling	The vertebrate retina: an approachable piece of the brain
Albert Szent-Györgyi	Biology of Donor-Acceptor interactions
Arthur Pardee	Biochemical studies in active transport
J. Woodland Hastings	The chemistry and biology of excited singlet oxygen
Darrell Fleischman	Luminescence and membrane potential in photo-synthetic bacteria
Henry Linschitz	Kinetic studies of photoreaction in phytochrome, chlorophyl and rhodopsin
Alvin Nason	Assimilatory nitrate reductase from *Neurospora*
Keith R. Porter	Microtubules in fish chromatophores
L. C. Junquira	
Ruth E. Benesch	Control mechanism for oxygen release by hemoglobin I
Reinholdt Benesch	Control mechanism for oxygen release by hemoglobin II
Sydney Brenner	Control mechanisms I
	Control mechanisms II
	Control mechanisms III
Alex Keynan	The outgrowing bacterial endospore as a system for the study of unicellular differentiation
John C. Eccles	Integration of information by cerebellar Purkinje cells
K. E. Van Holde	Pulsed-field electric dichroism of macromolecules
Guido Guidatti	Structure and function of hemoglobin
Klaus Weber	Structure of aspartic transcarbamylase
Eugene Kennedy	Membrane structure and function
Richard L. Sidman	Cell interaction in developing mouse brain
Edward A. Adelberg	DNA transfer and bacterial conjugation
David F. Waugh	Clotting mechanisms, implants and thrombosis

VIII. Special Seminars

Alan Weeds	The light chains of the myosin molecule
Joseph Ilan	Translational control of specific mRNA during insect metamorphosis

EXPERIMENTAL MARINE BOTANY

I. Consultants

Sterling B. Hendricks, U. S. Department of Agriculture
William S. Hillman, Brookhaven National Laboratory
Bessell Kok, Research Institute for Advanced Studies
Johan A. Hellebust, Harvard University

II. Instructors

Harold W. Siegelman, Plant Biochemist, Brookhaven National Laboratory, in charge of course
Trevor W. Goodwin, Professor of Biochemistry, University of Liverpool, England
Robert R. L. Guillard, Associate Scientist, Woods Hole Oceanographic Institution
Frank A. Loewus, Professor of Biology, State University of New York at Buffalo
John M. Olson, Biophysicist, Brookhaven National Laboratory
Robert T. Wilce, Associate Professor of Botany, University of Massachusetts

III. Special Lecturers

Lawrence Bogorad, Professor of Biology, Harvard University
Martin Gibbs, Professor of Biology, Brandeis University
Sarah Gibbs, Associate Professor of Botany, McGill University
Louis A. Hannic, Assistant Professor of Botany, Dalhousie University
John A. Hellebust, Associate Professor of Biology, Harvard University
Harvard Lyman, Associate Professor of Biology, State University of New York at Stony Brook
Gordon MacLachlan, Associate Professor of Botany, McGill University
Carl A. Price, Professor of Botany, Rutgers University
Joseph Ramus, Assistant Professor of Biology, Yale University
Jerome Schiff, Professor of Biology, Brandeis University

IV. Assistant and Collector

William A. McDaniel, Botany Department, University of Massachusetts

V. Lectures

H. W. Siegelman	Photochemically-active chromoproteins of plants
C. A. Price	Zonal centrifugation
John M. Olson	Photosynthesis—evolution, structure and function
J. A. Hellebust	Excretion of organics by phytoplankton
Frank A. Loewus	Structure and biosynthesis of plant cell walls and polysaccharides
Sarah Gibbs	Autoradiographic studies on RNA synthesis in chloroplasts
G. MacLachlan	Cellulose metabolism in relation to growth
T. W. Goodwin	Introduction to the carotenoids, steroids, and related substances
Harvard Lyman	Euglena I
R. R. L. Guillard	Nutritional requirements and productivity of phytoplankton
Martin Gibbs	Path of carbon in photosynthesis
L. A. Hanic	Plant cell wall with special reference to the algae

Max Bloomer	The role of hydrocarbons in the sea
L. Bogorad	Chloroplast development
Robert T. Wilce	Introduction to the major groups of benthic marine algae
J. Ramus	Aspects of the fine structure and developmental morphology of *Pseudogloiophloea* in culture
J. Schiff	Euglena II

EXPERIMENTAL INVERTEBRATE ZOOLOGY

I. Consultants

Frank A. Brown, Jr., Morrison Professor of Zoology, Northwestern University
C. Ladd Prosser, Professor of Physiology, University of Illinois
Clark P. Read, Professor of Biology, Rice University
Alfred C. Redfield, Woods Hole Oceanographic Institution
W. D. Russell-Hunter, Professor of Zoology, Syracuse University

II. Instructors

James F. Case, Professor of Biology, University of California, Santa Barbara, in charge of course
Frank M. Fisher, Jr., Associate Professor of Biology, Rice University
David C. Grant, Assistant Professor of Biology, Davidson College
Jonathan P. Green, Assistant Professor of Biology, Brown University
Michael J. Greenberg, Associate Professor of Biology, Florida State University
Robert K. Josephson, Associate Professor of Biology, Case Western Reserve University
Charlotte P. Mangum, Associate Professor of Biology, College of William and Mary
Alan Gelperin, Assistant Professor of Biology, Princeton University

III. Special Lecturers

Edward R. Baylor, Professor of Biology, State University of New York at Stony Brook
W. D. Russell-Hunter, Professor of Zoology, Syracuse University
Mahlon E. Kriebel, State University of New York, Upstate Medical Center
James G. Morin, Harvard University
C. Ladd Prosser, Professor of Physiology, University of Illinois

IV. Laboratory Assistants

Eldon E. Ball, University of California, Santa Barbara
Alan C. Eggleston, University of California, Santa Barbara
Roger C. Halverson, University of California, Santa Barbara

V. Lectures

H. Burr Steinbach	Introduction to the Marine Biological Laboratory
Robert K. Josephson	Coelenterata and Ctenophora
James F. Case	The study of invertebrates
Frank M. Fisher, Jr.,	Platyhelminthes, Aschelminthes, Nemertea, I and II
David C. Grant	The Cape Cod environment
James F. Case	Porifera

Charlotte P. Mangum	Annelids
Jonathan P. Green	Arthropoda, I and II
Michael J. Greenberg	Mollusca
W. D. Russell-Hunter	Ancestors and archetypes with reference to *Neopilina*
Alan Gelperin	Echinodermata
James F. Case	Protochordata
David C. Grant	Plankton
Frank M. Fisher, Jr.,	Cellular mechanisms of ingestion
	Ingestion mechanisms of multicellular animals
	Digestive physiology
Charlotte P. Mangum	Respiration: principles
C. Ladd Prosser	Thermal adaptation
Charlotte P. Mangum	Respiration: exchange mechanisms
	Respiration: transport mechanisms
David C. Grant	Community structure and diversity
Michael J. Greenberg	Circulation: some aspects of hemodynamics including flow in ciliated tubes
Mahlon E. Kriebel	Physiology of the tunicate heart
Michael J. Greenberg	Circulation: comparative physiology of the heart, ABRM and other muscles
Jonathan P. Green	Neuroendocrinology of annelids, arthropods and molluscs
	Physiology of the arthropod exoskeleton
	Osmoregulation
James F. Case	Luminescence
Edward R. Baylor	Stimuli associated with feeding behavior and aggregation of zooplankton in the sea
James F. Case	Sensory physiology: I
James G. Morin	*Obelia* luminescence
James F. Case	Sensory physiology: II
Robert K. Josephson	Behavior without brains: I. plants, protozoa, and sponges
	Behavior without brains: II, cnidarians
	Increasing neural complexity in lower metazoa
Alan Gelperin	Strategies of behavioral physiology
	Executive neurons
	Complex behavior in simple systems

MARINE ECOLOGY

I. Consultants

Melbourne R. Carriker, Director, Systematics-Ecology Program
Bostwick H. Ketchum, Woods Hole Oceanographic Institution
Edwin T. Moul, Rutgers University
John H. Ryther, Woods Hole Oceanographic Institution
Howard L. Sanders, Woods Hole Oceanographic Institution

II. Instructors

Lawrence B. Slobodkin, Department of Biology, State University of New York at Stony Brook, in charge of course

Edward R. Baylor, Department of Biology, State University of New York at Stony Brook

Sumner Richman, Department of Biology, Lawrence University
John Steele, Department of Agriculture and Fisheries, Aberdeen, Scotland
W. Rowland Taylor, Department of Earth and Planetary Sciences, The Johns Hopkins University and the Chesapeake Bay Institute
Edward O. Wilson, Department of Biology, Harvard University

III. Special Lecturer

Lev Fishelson, University of Tel Aviv, Israel

IV. Laboratory Assistants

Herman F. Bosch, Department of Earth and Planetary Sciences, The Johns Hopkins University
Wayne H. Bell, Department of Biology, Middlebury College

V. Lectures

Lawrence Slobodkin	Introduction to the course
	The politics of ecology
	Approaches to ecology
	The strategy of evolution
W. Rowland Taylor	The marine environment I. Chemistry of seawater
	The marine environment II. Solar radiation through seawater
	Phytoplankton I
	Phytoplankton II
	Primary productivity
S. Katona	Zooplankton
Sumner Richman	Introduction to ecological energetics
	Measurement of zooplankton feeding
	Factors affecting zooplankton feeding
Sumner Richman	Feeding of *Calanus* on synchronously growing cultures of diatoms
	Bomb calorimetry and respiration
Lawrence Slobodkin	Predation theory
	Classical models of ecological systems
Edward O. Wilson	Animal communication I
	Animal communication II
	Colonization and species equilibrium I
	Colonization and species equilibrium II
	Speciation I
	Speciation II
John Steele	North Sea food chains
	Chlorophyll and C^{14} uptake in sand
R. Mitchell	Antagonistic interactions between micro-organisms
John Steele	Pelagic food chains
	Oxygen uptake by sand
	Benthic food chains
	Energy flow in sand
	Theoretical models of food chains
	An experimental study of a marine food chain

Edward R. Baylor	Light-stimulated animal behavior Analysis of behavior by experiment and computer Cladoceran behavior—stimuli Cladoceran behavior—hunting and feeding Plankton patches and sea surface circulation I Plankton patches and sea surface circulation II
Howard L. Sanders	Physical and biological control of communities
Edward R. Baylor	Plankton patches and sea surface circulation III
Lev Fishelson	The ecology of coral reefs in the Gulf of Aquaba

SYSTEMATICS–ECOLOGY PROGRAM

The Staff

Director: Melbourne R. Carriker
Resident Systematist (Zoology): Robert P. Higgins
Acting Resident Systematist (Botany): Robert T. Wilce
Resident Ecologist: David K. Young
Assistant Ecologist: Katharine D. Hobson
Postdoctoral Fellows and Research Associates: David G. Cook, Celia R. Haigh, Lawrence R. McCloskey, Norman R. Sinclair, William C. Summers
Graduate Research Trainees: William R. Cobb, Joan R. Conway, Mary Ann Gilbert, William H. Gilbert, William D. Hummon, Jeffrey S. Levinton, Allan D. Michael, Leland W. Pollock, James R. Sears
Visiting Investigators in Residence: Edward L. Bousfield, Louise Bush, Martin A. Buzas, Howard H. Chauncey, Patricia L. Dudley, Marie U. Nylen, Joel S. O'Connor, Philip Person, Harold H. Plough, Donald C. Rhoads, Wesley N. Tiffney, Ruth D. Turner, Victor Z. Zullo
Consultants: William Randolph Taylor, Ruth D. Turner, Robert T. Wilce
Curator: Johanna M. Reinhart
Assistant Curator (Gray Museum Herbarium): Joan R. Conway
Technical Field Assistant: Peter J. Oldham
Field Assistant: Francis Doohan
Scientific Illustrators: Ruth von Arx, Susan P. Heller
Captain, R/V A. E. Verrill: James P. Ostergard
Mate, R/V A. E. Verrill: Peter Graham
Administrative Assistants: Hazel W. Fermino, Constance A. Brackett
Program Secretary: Eva S. Montiero
Research Assistants: Donald F. Allen, Anne C. Collins, Mary Ann Gilbert, William H. Gilbert, Gail Gingrass, Theodore J. Grant, David J. Hartzband, Marlyn J. Hummel, Richard A. McGrath, Charles R. McKay, John J. McMahon, Ellen P. McReynolds, Leland W. Pollock, Alfred Poor, Carol Q. Schwamb, Anne Smarsh, Michael A. Soukup, Martha Spiers, William Stamm, Richard Traverse, Linda Vacca, Dirk Van Zandt
Visitors: Mohamed Hyder, Pierre Lasserre, Barry A. Wade

Seminars (winter included)

Karl Banse	Oxygen consumption by the subtidal sea bed and the importance of the macrofauna
John S. Rankin, Jr.	Antarctic benthic communities
Celia R. Haigh	Polygamy in the redwinged blackbird
Norman R. Sinclair	Studies on the heterophyid trematodes *Apophallus brevis* and *A. imperator*
Robert R. Hessler	Deep sea isopods

William D. Hummon	Interspecific competition between gastrotrichs on several Woods Hole beaches
Sanford A. Moss	Heated effluents and the behavior of young shad exposed to thermal gradients
William Gosline	Some relationships between functional morphology and bony fish classification
Tom Fenchel	Aspects of research in interstitial ecology
James A. Slater	Aspects of biogeography in South Africa
James R. Sears	Aspects of life history and habitat ecology of sub-littoral benthic marine algae
Robert A. Murchelano	Bivalve larval culture—bacteriological considerations
David K. Young	Biogenic reworking of marine sediments
Joanna Z. Page	Control of reproduction in *Derbesia*
Wesley N. Tiffney, Jr.	Relationship between tree-line and snow cover in the White Mountains, N. H.
William C. Leggett	Observations on the migratory behavior of American shad in the Connecticut River utilizing ultrasonic tracking
Robert Marak	Determination of peak spawning times of haddock
Barry A. Wade	Kingston Harbor—a case study in conservation and development
Lawrence R. McCloskey	Ecology of an echinospira larvae
Philip B. Robertson	Larval development of scyllarid lobsters
R. Jack Schultz	Evolution of unisexuality and polyploidy in fish
Melbourne R. Carriker	Excavation of boreholes by the gastropod *Urosalpinx*: an analysis by means of light and scanning electron microscopy
Pierre Lassere	Distribution of meiobenthic oligochaetes as explained by physiological ecology
Joel O'Connor	Distribution of fishes over natural and sand-filled bottoms in a Long Island estuary
Norman Holme	Benthos study techniques
Robert L. Vadas	*Agarum* and the kelp bed community
Edward L. Bousfield	Adaptive radiation in sand-burrowing amphipod crustaceans
Victor A. Zullo	Temperate marine faunas: a paleontologist's view of the present
Donald C. Rhoads	The use of bivalves as environmental data recorders
David W. Folger	Land-derived biogenic particulates recovered from surface water and air during two Atlantic crossings
Lewis A. Hanic	Seaweed farming—problems and solutions
Ivan Valiela	Invasion and structuring of dung arthropod communities
Leland W. Pollock	Aspects of the ecology of interstitial tardigrades
Martin A. Buzas	Foraminiferal densities and environmental variables: the Linear model in marine ecology
John C. Russ	Characteristics and applications of the scanning electron microscope
Clay Sassaman	Aspects of temperature and low oxygen adaptations in coelenterates
M. Patricia Morse	Direct development in the nudibranch mollusc, *Coryphella salmonacea* Couthouy
Louise F. Bush	Small marine turbellaria of Cape Cod: some history, comments and questions

THE LABORATORY STAFF

Homer P. Smith, General Manager

Miss Jane Fessenden, Librarian

Carl O. Schweidenback, Manager, Supply Department

John J. Valois, Assistant Manager, Supply Department

Frank A. Wildes, Controller

Robert Kahler, Superintendent Buildings and Grounds

Robert Gunning, Assistant Superintendent, Buildings and Grounds

Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Mrs. Virginia R. Arthur
Edward J. Bender
Mrs. Florence S. Butz
Mrs. Colleen P. Hall
Mrs. Ann W. Loomis
Mrs. Vivian I. Manson
Miss Elaine C. Perry
Mrs. Cynthia S. Regan
Miss Mary Tavares

LIBRARY

Mrs. Virginia Brandenburg
Miss Elizabeth A. Brown
David J. Fitzgerald
Mrs. Lenora Joseph
Mrs. Doris Ricker

MAINTENANCE OF BUILDINGS AND GROUNDS

Eldon P. Allen
John T. Brady
Bernard F. Cavanaugh
Cecil Costa
John V. Day
Manuel P. Dutra
Charles Fuglister
Richard E. Geggatt, Jr.
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Kenneth J. Marks
Stephen A. Mills
Willy M. Neilson
Robert A. Serrano
Frederick E. Thrasher
Frederick E. Ward
Edward J. White

DEPARTMENT OF RESEARCH SERVICES

Gail M. Cavanaugh
Lowell V. Martin
Mrs. Juliette T. Lauzon
Frank E. Sylvia

SUPPLY DEPARTMENT

Courtney Barber, III
David H. Graham
Robert Hampton
Lewis M. Lawday
Robert O. Lehy
Miss Joyce B. Lima
Eugene Tassinari
Bruno F. Trapasso
John Varao

DINNING HALL AND HOUSING

Robert T. Martin, Manager, Food Service
Norman Michaud, Head Chef
Mrs. Elizabeth Kuil, Supervisor, Dining Room
Mrs. Ellen T. Nickelson, Supervisor, Dormitories
Alan G. Lunn, Supervisor, Cottage Colony

3. Investigators: Lillie, Grass and Rand Fellows; Students

Independent Investigators, 1969

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Piper, Judith Ann, University of Connecticut
Poor, Alfred, Systematics-Ecology Program
Poston, Robin, Duke University
Prusch, Robert D., Syracuse University
Ravitz, Melvyn Jay, Albert Einstein College of Medicine
Rayner, Ellen P., Brandeis University
Reinhart, Johanna M., Systematics-Ecology Program
Riggio, Bonnie L., University of Massachusetts
Roark, Dennis E., University of Connecticut
Robertson, Lola E., American Museum of Natural History
Rodewald, Richard D., University of Pennsylvania
Roessner, Karen D., Case Western Reserve University
Rorke, Charles, Wistar Institute of Anatomy and Biology
Rose, Birgit, College of Physicians and Surgeons, Columbia University
Rowen, Adam J., Yale University
Rubinstein, Neal A., Dartmouth College
Sachs, Martin, University of Massachusetts
Sage, Jean A., Indiana University Medical Center
Salmon, Edward, University of Pennsylvania
Sandlin, Ronald A., National Institutes of Health
Sassaman, Clay, College of William and Mary
Saul, Richard, University of Maryland
Schmidlin, Annelise, Princeton University
Schwartzman, Joseph D., Dartmouth College
Sears, James R., Systematics-Ecology Program
Shepard, Charles A., Oberlin College
Shiroky, Dorothy V., The Johns Hopkins University
Singer, Irwin I., New York University
Slaughter, Margaret Ann, Yale University School of Medicine
Slavin, William R., University of Connecticut
Smarsh, Anne, Systematics-Ecology Program
Sogin, Stephen J., University of Illinois
Soukup, Michael A., Systematics-Ecology Program
Stafford, Walter, University of Connecticut
Stamm, William C., Jr., Drew University
Starling, Jane A., Rice University
Stewart, James R., Brown University
Straus, Andrea, Kirkland College
Streibel, Ellen, University of Pennsylvania
Suddith, Robert L., Indiana University
Sussman, Joel L., Columbia University

Szamier, Robert B., Albert Einstein College of Medicine
Szonyi, Eszter, Institute for Muscle Research
Tang, Cecilia, University of Connecticut
Tann, Edward M., Ohio Dominican College
Tatum, Susan M., University of Pennsylvania
Terebey, Nicholas, State University of New York, Upstate Medical Center
Theile, Judith A., Rensselaer Polytechnic Institute
Thieme, Elisabeth, Rutgers University
Truchan, Sister Leona, Northwestern University
Tucker, Gail S., University of Kansas
Turner, Robert S., Jr., University of Oregon
Van Wie, Donald G., Harvard University
Van Zandt, Dirk, Systematics-Ecology Program
Wexler, Andrew M., Phillips Academy
Wideman, Cyrilla, Illinois Institute of Technology
Williams, Lois D., University of Hawaii
Wilson, David S., Brandeis University
Wolley, Robert C., Tulane University
Wolsey, Penny, McGill University, Canada
Woodcock, Frances J., University of Miami
Wozenski, Janet, Syracuse University
Yee, Ann G., Harvard University
Young, Janice E., Northwestern University
Zakevicius, Jane M., New York University Medical Center
Zavitkovski, Jerry, Oregon State University
Zipser, Birgit, Albert Einstein College of Medicine

Library Readers, 1969

Allen, Garland E., Assistant Professor of Biology, Washington University
Anderson, Rubert S., Woods Hole, Massachusetts
Appel, Antoinette R., Queens College
Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Berlin, Richard D., Assistant Professor of Physiology, Harvard Medical School
Berne, Robert M., Chairman and Professor of Physiology, University of Virginia School of Medicine
Bodansky, Oscar, Vice President, Sloan-Kettering Institute for Cancer Research
Boettiger, Edward G., Professor of Physiology, University of Connecticut
Carlson, Francis D., Professor of Biophysics, The Johns Hopkins University
Chase, Aurin M., Emeritus and Senior Research Biologist, Princeton University
Crane, Robert K., Chairman and Professor of Physiology, Rutgers Medical School
Davis, John D., Assistant Professor of Biological Sciences, Smith College
De Robertis, Eduardo, Professor and Director of Institute, University of Buenos Aires
Duboff, Gregory S., Professor of Experimental Medicine, University of Michigan School of Medicine
Ebert, James, Director and Professor of Embryology, Carnegie Institution of Washington
Edds, M. V., Jr., Professor of Biology, Brown University
Flavin, Rev. John W., Chairman, Department of Biology, College of the Holy Cross
Gabriel, Mordecai L., Chairman and Professor of Biology, Brooklyn College
German, James, Investigator and Director of Laboratory of Human Genetics, The New York Blood Center
Gibbs, Martin, Professor of Biology, Brandeis University
Gitlin, David, Professor of Pediatrics, University of Pittsburgh School of Medicine
Goudsmit, Esther M., Research Associate, The Johns Hopkins University
Grant, Robert J., Assistant Professor of Biological Sciences, Hunter College
Green, James W., Professor of Physiology, Rutgers University
Handler, Philip, Professor of Biochemistry, Duke University

Hubbard, Susan Baur, Marine Biological Laboratory
Isselbacher, Kurt J., Professor of Medicine, Massachusetts General Hospital
Kaltenbach, Jane C., Associate Professor of Biological Sciences, Mount Holyoke College
Kane, Robert E., Assistant Director and Associate Professor Pacific Biomedical Research Center, University of Hawaii
Kempton, Rudolf T., Professor Emeritus of Biology, Vassar College
Keosian, John, Professor of Biochemistry, Rutgers University
Kravitz, Edward A., Professor of Neurobiology, Harvard Medical School
Levinthal, Cyrus, Chairman, Department of Biological Sciences, Columbia University
Lochhead, John H., Professor of Zoology, University of Vermont
Mahler, Henry R., Professor of Chemistry, Indiana University
Marshak, Alrfed, Professor of Experimental Pathology, Tulane University
Nachmansohn, David, Professor of Biochemistry, Columbia University, College of Physicians and Surgeons
Nasatir, Maimon, Chairman and Professor of Biology, University of Toledo
Osterhout, Marion L., Marine Biological Laboratory
Ravin, Herbert A., Director and Professor of Medicine, Sinai Hospital of Detroit and Wayne State University
Rosenbluth, Jack, Associate Professor of Physiology and Biophysics, New York University College of Medicine
Rowland, Lewis P., Chairman and Professor of Neurology, University of Pennsylvania
Rubinow, Sol I., Professor of Biomathematics, Cornell University Medical College
Sager, Ruth, Professor of Biological Sciences, Hunter College
Schlesinger, R. Walter, Chairman, Department of Microbiology, Rutgers University
Scott, Alan, Professor of Biology, Colby College
Sirlin, J. L., Associate Professor of Anatomy, Cornell University Medical College
Smelser, George K., Director of Eye Research, College of Physicians and Surgeons, Columbia University
Sonnenblick, B. P., Professor of Zoology and Radiation Science, Rutgers University
Spector, Abraham, Professor of Ophthalmology, College of Physicians and Surgeons, Columbia University
Sperelakis, Nick, Professor of Physiology, University of Virginia School of Medicine
Stetten, DeWitt, Jr., Dean and Professor of Experimental Medicine, Rutgers Medical School
Stetten, Marjorie R., Research Professor of Experimental Medicine, Rutgers Medical School
Stracher, Alfred, Professor of Biochemistry, State University of New York, Downstate Medical Center
Trotter, Philip J., Interdisciplinary Postdoctoral Fellow, New England Institute, Inc.
Vagelos, P. Roy, Chairman and Professor of Biochemistry, Washington University School of Medicine
Wainio, Walter, Chairman and Professor of Biochemistry, Rutgers University
Waksman, Byron H., Chairman and Professor of Microbiology, Yale University
Weiss, Leon, Professor of Anatomy, The Johns Hopkins University Medical School
Wheeler, George E., Associate Professor of Biology, Brooklyn College
Whiting, Anna R., Consultant, Biology Division, Oak Ridge National Laboratory
Wichterman, Ralph, Professor of Biology, Temple University
Wilson, T. Hastings, Chairman and Professor of Physiology, Harvard Medical School
Wittenberg, Jonathan B., Professor of Physiology, Albert Einstein College of Medicine
Yow, Frank W., Professor of Biology, Kenyon College
Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center
Zacks, Sumner I., Associate Professor of Pathology, University of Pennsylvania
Zeidenberg, Phillip, Senior Research Psychiatrist, New York State Psychiatric Institute
Zipser, David, Assistant Professor of Molecular Biology, Columbia University

Students, 1969

All students listed completed the formal course program, June 16–July 26. Asterisk indicates students completing post-course reaserch program, July 27–August 30.

ECOLOGY

Arnett, Patricia M., Indiana State University
*Bloom, Stephen A., University of South Florida
Brothers, Edward B., Scripps Institution of Oceanography
Flessa, Karl W., Brown University
*Gleye, Linda G., Sarah Lawrence College
*Permesly, Lester S., Tufts University School of Medicine
Quick, Frank W., Jr., University of Louisville
*Rex, Michael A., Harvard University
Schultz, John C., University of Chicago
*Shimek, Ronald L., Montana State University
*Slozak, Frances, University of Massachusetts
*Smith, Sharon L., College of St. Teresa
*Weinstein, Judith D., University of Massachusetts
*Williams, Ernest H., Jr., Princeton University
Williamson, Janet L., Oberlin College
Wing, Daniel C., Oberlin College

EMBRYOLOGY

*Bacheler, Lee T., The Johns Hopkins University
*Broyles, Robert H., The Bowman Gray School of Medicine
Buckley, Patricia A., University of Virginia
Callaway, Edward E., University of California, Davis
*Cameron, Robert A., University of California, Santa Cruz
*Conway, Arthur F., University of Miami
*Desmond, Mary E., University of Colorado
Gordon, Richard, Columbia University
Hendrickson, Scott A., Washington University
*Hofman, Florence C., University of Wisconsin
Klayman, Michael B., Union College
Lipton, Bruce H., University of Virginia
*Packard, David S., Jr., Louisiana State University
*Sentkowski, Alan M., The Johns Hopkins University
*Shermoen, Antony W., Wesleyan University
*Thistlethwaite, J. Richard, Amherst College
Tischfield, Jay A., Yale University
*Toole, Bryan P., Massachusetts General Hospital
*Wieschaus, Eric F., University of Notre Dame
*Wolin, Edward M., Reed College

EXPERIMENTAL BOTANY

*Burr, Judith E., Drew University
Campbell, Robert M., University of St. Andrews, Scotland
*Diner, Brian A., The Rockefeller University
*Groves, Samuel T., Yale University
*Hughes, Gloria J., University of Rhode Island
Kamykowski, Daniel L., Scripps Institution of Oceanography
*Kent, Graham Ronald, University of Guelph, Canada
*Levi, Carolyn A., University of Massachusetts
McCormick, Jon M., Millersville State College
*McDaniel, William A., University of Massachusetts
*Moshier, Suzanne E., University of Chicago
*Mozgala, Walter J., University of Massachusetts
Nielson, Andrea L., University of Massachusetts
*Owens, Olga V., University of Maryland

*Sperling, Patricia G., Kent State College
Williams, Nina S., University of Maryland

PHYSIOLOGY

*Andrews, Thomas G., Jr., College of Physicians and Surgeons, Columbia University
*Bleich, Hermann E., Columbia University
*Brackenbury, Robert W., California Institute of Technology
*Bronk, Burt V., Queens College
*Chambliss, Glenn H., University of Chicago
*Collins, Terryl R., Simon Fraser University, Canada
*Daniel, James L., Carnegie-Mellon University
*Epstein, Paul M., University of Massachusetts
*Fleming, Honoree, University of Chicago
*Goldsmith, Melvin, University of Connecticut
*Hsi, Edward S., University of Minnesota
Kovacs, Mark A., Massachusetts Institute of Technology
*Lehman, William J., Princeton University
*Li, Jade, Bryn Mawr College
*Lucas, Roger C., Illinois Institute of Technology
*Manson, Michael, The Johns Hopkins University
*Mao, Chi-Chiang, University of Oklahoma
*Messer, Anne, University of Oregon
Nist, Cynthia R., The Rockefeller University
Paspy, Terry L., University of Illinois, Urbana
*Rudd, Jennifer N., Wesleyan University
*Spolsky, Christina M., Yale University
Straley, Susan C., Cornell University
*Szuts, Ete Z., The Johns Hopkins University
*Teichberg, Saul, Columbia University
*Waters, Coreen A., Brandeis University
*Waterston, Robert H., University of Chicago
*Weiss, Howard D., Northwestern University Medical School
*Wood, Donald S., Washington State University
*Wood, Kathleen A., Washington State University
Yonath, Jacob, Weizmann Institution, Rehovoth

INVERTEBRATE ZOOLOGY

Billingsley, Brynder C., Tougaloo College
Caldarola, Patricia, Cornell University
Casaday, George B., Cornell University
Chaiken, Michael B., State University of New York at Stony Brook
Chin, Kenneth W., Columbia University
*Cowan, Philip E., Glasgow University, Scotalnd
*Donaldson, Sven, New College, Sarasota
*Eggleston, Linda L., Rice University
Ellsworth, Janet P., State University of New York Upstate Medical Center
Fano, Virginia, University of Wisconsin, Madison
Foelix, Ranier F., National Science Foundation Department of Mental Health, Raleigh
Goodman, Mark L., University of Massachusetts, Amherst
*Haberfield, Eve C., University of Rhode Island
*Hackett, John T., University of Illinois
Inhaber, Francine, City College of New York
*Irwin, George H., III, Emory University
Jungreis, Arthur M., University of Minnesota
*Kahler, George A., III, Rice University
*McArdle, Florence E., Columbia University

*MIYAMOTO, DAVID M., University of California, San Diego
NORELLI, LINDA K., Bryn Mawr College
ODESSBY, RICHARD, Harvard University
PEZALLA, PAUL D., University of Minnesota
*PRIOR, DAVID J., University of Virginia
*RAM, JEFFREY L., California Institute of Technology
*REHM, JANET N., Scripps Institution of Oceanography
ROHRLICH, SUSANNAH T., Harvard University
*SEGUIN, LANA R., Indiana University
*SKARF, BARRY, The Johns Hopkins University
STAMM, WILLIAM C., JR., Drew University
TEREBEY, NICHOLAS, State University of New York, Upstate Medical Center
TESTERMAN, JOHN K., University of California, Irvine
VACCA, LINDA LEE, University of Southern California
*WESTON, JOANNE, Cornell University
WILSON, SAMUEL, Scripps Institution of Oceanography

4. FELLOWSHIPS AND SCHOLARSHIPS, 1969

The Lucretia Crocker Scholarship:
NINA S. WILLIAMS, Experimental Botany Course
The James Watt Mavor Scholarship:
ROBERT M. CAMPBELL, Experimental Botany Course
PHILIP E. COWAN, Invertebrate Zoology Course
The Edwin Grant Conklin Scholarship:
BRYON P. TOOLE, Embryology Course

5. TRAINING PROGRAMS

FERTILIZATION AND GAMETE PHYSIOLOGY RESEARCH TRAINING PROGRAM

I. INSTRUCTORS
CHARLES B. METZ, University of Miami, Program Chairman
JOHN D. BIGGERS, The Johns Hopkins University
MARCO CRIPPA, University of Palmero, Italy
GERTRUDE W. HINSCH, University of Miami
KURT KÖHLER, University of Montpellier, France
LEONARD NELSON, MEDICAL College of Ohio at Toledo

II. CONSULTANTS
JEAN CLARK DAN, Ochanomizu University, Tokyo
PAUL GROSS, Massachusetts Institute of Technology
CLAUDE VILLEE, Harvard Medical School

III. LABORATORY ASSISTANTS
MARILYN L. CAYER, Electron Microscope Assistant
LAURA HOSKIN, Secretary
FRANCES WOODCOCK, Photographic Assistant

IV. TRAINEES
BUTZEK, JAMES C., Illinois Institute of Technology
CLARK, WALLIS H., University of California, Riverside
CONWAY, CAROLYN M., University of Miami
COOMES, ROGER K., Monkato State College
ELINSON, RICHARD P., Yale University
FENWICK, RAYMOND G., JR., Miami University
FORTUNE, JOANNE E., Cornell University
HOPPER, JAMES E., University of Wisconsin

Koser, Richard B., Brooklyn College
Lavin, Lora H., Rosemont College
Lee, Harold H., University of Toledo
O'Melia, Anne F., Fordham University
Sacco, Anthony G., University of Tennessee
Schroeder, Paul C., Washington State University
Shelley, Thomas F., State University of New York, Upstate Medical Center
Weinberg, Eric S., The Rockefeller University

V. Lectures

Kurt Köhler	The passage of mRNA from the nucleus to cytoplasm
R. M. Iverson	RNA synthesis during oogenesis and development
W. J. Humphreys	Structure and function of cortical granules in invertebrate eggs
C. J. Brokow	Recent studies on sperm mobility
L. E. Franklin	The acrosome reaction and sperm penetration in the golden hamster egg
M. Crippa	Mechanisms of control of ribosomal RNA synthesis during Xenopus oogenesis
Mildred Gordon	Cytochemical analyses of mammalian sperm flagella
J. Gall	Differential gene synthesis during oogenesis
H. A. Bern	Vertebrate prolactin: a reproductive hormone with non-reproductive beginnings

Excitable Membrane Physiology and Biophysics Training Program

I. Instructors

William J. Adelman, Jr., University of Maryland School of Medicine, Program Chairman
John W. Moore, Duke University School of Medicine
Toshio Narahashi, Duke University School of Medicine
Yoram Palti, Hebrew University, Hadassah Medical School
Tobias Schwartz, University of Connecticut

II. Consultants

Kenneth S. Cole, National Institutes of Health
Harry Grundfest, College of Physicians and Surgeons, Columbia University
Lorin J. Mullins, University of Maryland School of Medicine
Ichiji Tasaki, National Institutes of Health

III. Trainees

Callahan, Leo X., University of Maryland
Cleaves, Carol Ann, Duke University
Davis, Donald G., University of Pittsburgh
Dawson, David C., University of Pittsburgh
De Hemptinne, Alex, Duke University
Di Polo, Reinaldo, Harvard University Medical School
Ernau, Mildred C., State University of New York at Albany
Jendrasiak, Gordon L., University of Notre Dame
Jones, Cedron, Massachusetts Institute of Technology
Korenbrot, Juan I., The Johns Hopkins University
McCreery, Douglas B., University of Connecticut
Waggoner, Alan S., University of Oregon

IV. Lectures

H. Burr Steinbach	Introduction
Tobias Schwartz	The flux equation
	The Ussing-Teorell "unidirectional flux ratio"—osmotic equilibrium—the Staverman reflexion coefficient
	The Gibbs-Donnan equilibrium
	The Goldman equation
	Simple diffusion regimes—electrical equivalent circuits

Raymond A. Sjodin	Struction of cell membranes
	Diffusion and permeation in cell membranes
	Radioactive tracers and analysis of kinetics
	Results: nerve, muscle and erythrocytes
	Active ion transport
Lorin J. Mullins	Active transport techniques in squid axons
	Active transport: stoichiometry, substrate requirements and electrical consequences
	Models for active and passive ion transport
	Models for nerve excitation
Kenneth S. Cole	Passive membrane properties I
	Passive membrane properties II
C. R. Worthington	X-ray analysis of nerve myelin
	Myelin structure
I. Stillman	Preparation of synthetic lipid bilayers
	K-selective macrocyclic polypeptides
Kenneth S. Cole	Voltage clamp strategy
John W. Moore	Voltage clamp technique: normal currents
	Ionic conductance kinetics: Hodgkin-Huxley equations
	Specifications for nerve membrane models
Daniel L. Gilbert	Fixed surface charges
Yoram Palti	Varying potential control voltage clamp
	Reconstruction of the membrane action potential
	Reconstruction of the propagated action potential and excitation phenomena
R. FitzHugh	Phase-space analysis of nerve models
	Dimensional analysis of nerve models
	Impulse propagation in a nerve fiber (film)
Daniel L. Gilbert	Internal perfusion of squid axons: techniques
William J. Adelman	Internal perfusion of squid axons
	Internal perfusion: voltage clamp results
	Membrane asymmetries
Harvey Fishman	Voltage clamp studies of frog skin
Rita Guttman	Temperature and nerve membrane function
Toshio Narahashi	Polyvalent cations in nerve excitation
	Membrane conductance and drug action I—tetrodotoxin
	Membrane conductance and drug action II—insecticides and tetraethylammonium
Robert L. De Haan	Electrical recording from single heart cells isolated in tissue culture
B. Hille	Voltage clamp studies of frog myelinated axons
	Specifications for an ionic channel
Lawrence Cohen	Light scattering and birefringence changes during nerve activity
Ichiji Tasaki	Internal perfusion studies of the squid axon
	Electrophysiological properties of excitable membranes
	Optical properties of excitable membranes
Harry Grundfest	Varieties of excitable membranes
	General ionic requirements for electrogenesis
	Electric organs
D. E. Goldman	Model making: general principles
	Basic physical mechanisms in excitability
	Survey of current model systems
	A detailed model of membrane excitability

V. WORKSHOPS

Paul De Weer	Isotopic techniques
Yoram Palti	Digital computer analysis and reconstruction of nerve behavior

6. TABULAR VIEW OF ATTENDANCE, 1965–1969

	1965	*1966*	*1967*	*1968*	*1969*
INVESTIGATORS—TOTAL	572	555	590	528	566
Independent	284	287	313	281	310
Library Reader	62	77	78	76	68
Research Assistants	227	191	199	171	188
STUDENTS—TOTAL	128	126	132	122	118
Invertebrate Zoology	41	37	41	39	35
Embryology	20	22	20	20	20
Physiology	30	29	31	30	30
Experimental Botany	20	18	20	15	16
Ecology	17	20	20	18	17
TRAINEES—TOTAL	16	16	16	17	29
TOTAL ATTENDANCE	734	710	738	667	708
Less persons represented in two categories	4	0	4	7	5
	730	710	734	660	703
INSTITUTIONS REPRESENTED—TOTAL	218	198	177	169	187
FOREIGN INSTITUTIONS REPRESENTED	27	28	29	23	24

7. INSTITUTIONS REPRESENTED, 1969

Albany Medical School
Albert Einstein College of Medicine
American Museum of Natural History
Aquarium Systems, Inc.
Barnard College
Baylor University College of Medicine
Boston University
Brandeis University
Brookhaven National Laboratory
Brooklyn College, The City University of New York
Brown University
Bryn Mawr College
California Institute of Technology
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, San Francisco
California, University of, Santa Barbara
California, University of, Santa Cruz
Carnegie Institution of Washington
Case Western Reserve University
Case Western Reserve University Medical School
Chicago, University of
Children's Cancer Research Foundation
Cincinnati, University of
City College of New York, The
Colby College
College of the Holy Cross
College of Physicians and Surgeons, Columbia University
College of William and Mary
Colorado, University of
Colorado, University of, Medical Center
Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut, University of
Connecticut, University of, Health Center
Connecticut, University of, Medical School
Connecticut, University of, School of Pharmacy
Cornell University
Cornell University Medical College
Dartmouth College
Davidson College
Delaware, University of
Drew University
Duke University
Duke University Medical Center
Emory University
Florida, University of
Florida State University
Fordham University
George Washington University School of Medicine
Goucher College

Harvard Medical School
Harvard University
Hawaii, University of
Hudson Valley Community College
Hunter College
Illinois, University of
Illinois Institute of Technology
Indiana State University
Indiana University
Institute for Cancer Research, The
Institute for Muscle Research, The
Institute of Molecular Evolution, University of Miami
Institute of Psychiatric Research, Indiana University Medical Center
Iowa, University of
John Carroll University
Johns Hopkins University, The
Johns Hopkins University, The, School of Hygiene
Johns Hopkins University, The, School of Medicine
Juniata College
Kansas, University of
Kent State College
Kentucky, University of
Kenyon College
Kettering, Charles F., Research Laboratory
Kirkland College
Lawrence University
Lehigh University
Louisiana State University
Louisville, University of
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of
Massachusetts General Hospital
Massachusetts Institute of Technology
Medical College of Ohio at Toledo
Mellon Institute of the Carnegie-Mellon University
Miami, University of
Miami University
Michigan, University of, School of Medicine
Michigan, University of
Michigan State University
Millersville State College
Minnesota, University of
Monkato State College
Montana State University
Mount Holyoke College
National Cancer Institute
National Institutes of Health
National Science Foundation
Naval Undersea Warfare Center
New College, Sarasota
New England Institute, Inc.
New York Blood Center, The
New York State Psychiatric Institute
New York University
New York University College of Dentistry
New York University Medical College
New York University School of Medicine
North Carolina, University of
North Carolina State University, Raleigh
North Dakota, University of
Northeastern Illinois State College
Northwestern University
Northwestern University Medical School
Notre Dame, University of
Oak Ridge National Laboratory
Oberlin College
Ohio Dominican College
Oklahoma, University of
Oregon, University of
Oregon State University
Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Pennsylvania Hospital
Pittsburgh, University of
Pittsburgh, University of, School of Medicine
Princeton University
Providence College
Queens College, The City University of New York
Reed College
Rensselaer Polytechnic Institute
Rhode Island, University of
Rhode Island Hospital
Rice University
Rochester, University of
Rochester, University of, Medical School
Rockefeller University, The
Rosemont College
Rutgers University
Rutgers University Medical School
St. Louis University
St. Teresa, College of
Scripps Institution of Oceanography
Simon Fraser University
Sinai Hospital of Detroit
Sloan-Kettering Institute for Cancer Research
Smith College
Smithsonian Institution of Washington
South Florida, University of
Stanford University
State University of Iowa
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
State University of New York at Albany
State University of New York at Buffalo
State University of New York at Stony Brook

Syracuse University
Temple University
Tennessee, University of
Texas, University of, Austin
Toledo, University of
Tougaloo College
Trinity College
Tufts University School of Medicine
Tulane University
Vanderbilt University School of Medicine
Vassar College
Vermont, University of
Veterans Administration Central Office, Washington, D. C.
Veterans Administration Hospital, Brooklyn
Veterans Administration Hospital, Pittsburgh
Virginia, University of
Virginia, University of, School of Medicine
Washington State University
Washington University
Washington University School of Medicine
Wayne State University
Wesleyan University
Wisconsin, University of
Wistar Institute of Anatomy and Biology
Woods Hole Oceanographic Institution
Yale University
Yale University School of Medicine

Foreign Institutions Represented, 1969

Bristol, University of, England
Buenos Aires, University of, Argentina
Centre National de la Recherche Scientifique, France
Frankfurt, University of, Germany
Glasgow, University of, Scotland
Guelph, University of, Canada
Hebrew University Medical School, Jerusalem
Instituto Venezolano de Investigaciones Científicas, Venezuela
Laboratory of Molecular Biology, England
Marine Laboratory, Aberdeen, Scotland
McGill University, Canada
Medical Research Council, England
Montreal, University of, Canada
N. Copernicus University, Poland
Ocean Research Institute, University of Tokyo, Japan
Ochanomizu University, Japan
Ottawa, University of, Canada
Palermo, University of, Italy
Research Institute of National Defence, Sweden
Simon Fraser University, Canada
St. Andrews, University of, Scotland
Tokyo, University of, Japan
Toronto, University of, Canada
Weizmann Institution, Israel

8. Friday Evening Lectures, 1969

July 4

Trevor W. Goodwin............Stereospecific studies on biosynthesis of carotenoids and sterols in plants
University of Liverpool

July 11

Jean Clark Dan...............The acrosomal process membrane
Ochanomizu University, Tokyo
Lillie Fellow at the MBL

July 17

Eduardo De Robertis..........Synthesis, storage and release of transmitter substances
University of Buenos Aires
Alexander Forbes Lecturer at MBL
Senior Grass Fellow at the MBL

July 18

Eduardo De Robertis..........Isolation and nature of chemical receptors

July 25

John H. Steele................Experimental studies of marine food chains
Marine Laboratory
Aberdeen, Scotland

August 1

Hugh E. Huxley Medical Research Council Cambridge, England	The design requirements of striated muscle

August 8

Martin Lindauer University of Frankfurt, Germany Rand Fellow at the MBL	Learning and forgetting in honeybees

August 15

J. Woodland Hastings Harvard University	Living light: biochemical mechanisms and biological function of bioluminescence

August 22

Sydney Brenner Medical Research Council Cambridge, England	Prospects for the complete analysis of the nervous system of nematodes

9. Tuesday Evening Seminars, 1969

July 15

S. Yuyama Arthur M. Zimmerman	Temperature—pressure effects on RNA synthesis in synchronized *Tetrahymena*
Haruo Kanatani	Effects of adenine derivatives on oocyte maturation and spawning in starfish
Arline C. Schmeer, O.P.	*Mercenaria* (clam) extracts: effect on hela cytokinetics
Catherine Henley D. P. Costello M. B. Thomas W. D. Newton	The 9 + 1 pattern of microtubules in spermatozoa of certain Turbellaria

July 22

Roger Milkman	An exact study of polygenic variation in natural populations
A. M. Jungreis	Changes in metabolite regulation as functions of acclimation temperature, starvation and season in the frog *Rana pipiens*
L. T. Graham, Jr.	Dark adaptation: its influence on GABA in frog retina, *in vivo*

July 29

Seymour Zigman	Variations in lens insoluble protein
R. Rodewald	Selective antibody transport in the proximal small intestine of the neonatal rat
P. G. Sokolove	Role of sodium pump in stretch receptor adaptations
John D. Palmer H. B. Dowse	Preliminary findings on the effect of D_2O on the period of circadian activity rhythms

August 5

W. J. Deal	Photoregulation of bioelectricity by photochromic acetylcholine receptor inhibitors
Henry G. Mautner E. Shefter	Studies of the conformation of small molecules acting on acetylcholine receptors
M. J Greenberg	Some properties of junctional cilia in molluscan gills

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F. C. G. Hoskin	Possible significance of "DFP-ASE" in squid nerve
Tobias L. Schwartz	The Goldman equation: its constraints and its applicability in the face of electrogenic and non-electrogenic pumps
A. L. Pearlman N. W. Daw	Color opponent cells in cat lateral geniculate nucleus
C. K. Bartell M. Fingerman	Application of continuous particle electrophoresis for the separation of lipoproteins having melanin-dispersing activity from eyestalks of fiddler crabs (*Uca pugilator*)

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V. REPORT OF THE LIBRARIAN

The space problem in the stack area of the library has been solved temporarily. The reprints have been compressed and will be placed in the basement stack leaving no room for expansion. One entire floor of stacks is now available to the serials section, relieving the growth situation for the next few years. Unfortunately this move means that we will be unable to accept any reprints until more floor area is added to the library.

During 1969 we received 4563 requests for articles contained in the library. We made 370 requests to other libraries for use by investigators here. 2,076 volumes were sent to the bindery and holdings now total 140,111. This figure does not include the reprint collection.

Total Number of Serial Titles	4,054
Number received currently	2,384
(On subscription	958
On exchange	981
On gift basis	365)
Number of text books added	302
(Received from book exhibit	160)
Reprints added in 1969	1,106

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1969, amounted to $2,197,603 and the corresponding securities are entered in the books at a value of $1,574,735. This compares with values of $2,403,484 and $1,575,096, respectively, at the end of the preceding year. The average yield on the securities was 4.37% of the market value and 6.09% of the book value. Uninvested principal cash was $1,104. Classification of the securities held in the Endowment Fund appears in the Auditor's Summary of Investments.

The market value of the Pooled Securities at December 31, 1969, amounted to $763,700 as compared to book values of $634,152. These figures compare with values of $824,502 and $666,202, respectively, at the close of the preceding year. The average yield on the securities was 3.57% of the market value and 4.30% of the book value. Uninvested principal cash was in the amount of $447.

The proportionate interest in the Pool Fund Account of the various funds, as of December 31, 1969, is as follows:

Pension Funds	23.048%
General Laboratory Investment	20.659%
F. R. Lillie Memorial Fund	2.252%
Anonymous Gift	.772%
Other:	
Bio Club Scholarship Fund	.587%
Rev. Arsenius Boyer Scholarship Fund	.709%
Gary N. Calkins Fund	.671%
Allen R. Memhard Fund	.129%
Lucretia Crocker Fund	2.438%
E. G. Conklin Fund	.411%
Jewett Memorial Fund	.217%
M. H. Jacobs Scholarship Fund	.294%
Herbert W. Rand Fellowship	20.834%
Mellon Foundation	9.835%
Mary Rogick Fund	2.157%
Swope Foundation	5.412%
Clowes Fund	9.575%

Donations from MBL Associates for 1969 amounted to $9,246 as compared with $8,360 for 1968. Unrestricted gifts from foundations, societies and companies amounted to $18,065.

During the year we administered the following grants and contracts:

Investigators	*Training*	*MBL Institutional*
10 NIH	3 NIH	2 NIH
3 NSF	1 NSF	2 NSF
1 ONR		1 AEC
1 Whitehall		1 Ford
15	4	6

An overhead rate of 20% of allowable direct costs was still in effect for most federally funded research grants at the beginning of the year, but this rate was generally superseded by the new rate of 25% which had been negotiated. However, Federal grants now require a cost sharing contribution by the Laboratory to research projects, usually at a rate of about 5%. An indirect cost rate based on the laboratory space allocated for a research project has now been negotiated with the federal agencies, for grants initiated after August 1, 1969.

The following is a statement of the auditors:

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts

> We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1969, the related statement of operating expenditures and income and statement of funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We examined and have reported on financial statements of theLaboratory for the year ended December 31, 1968.
>
> In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1969 and 1968 and the results of its operations for the years then ended on a consistent basis.
>
> The supplementary schedules included in this report were obtained from the Laboratory's records in the course of our examination and, in our opinion, are fairly stated in all material respects in relation to the financial statements, taken as a whole.
>
> Boston, Massachusetts
> March 20, 1970 LYBRAND, ROSS BROS. AND MONTGOMERY

It will be noted from the operating statement that the Laboratory activities for the year under review, amounted to a figure of a little over 1.5 million dollars, which amount is comparable to last years results.

ALEXANDER T. DAIGNAULT,
Treasurer

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1969 and 1968

Investments

	1969	*1968*
Investments held by Trustee:		
Securities, at cost (approximate market quotation 1969—$2,197,603)	$1,574,735	$1,575,096
Cash	1,104	624
	1,575,839	1,575,720
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation 1969—$763,700) less $5,728 temporary investment of current fund cash	628,424	660,474
Other investments	1,474,150	2,282,150
Cash	447	7,653
Accounts receivable	33	33
Due from current fund	61,622	53,276
	$3,740,515	$4,579,306

Plant Assets

	1969	*1968*
Land, buildings, library and equipment	6,072,007	5,732,782
Less allowance for depreciation (note 1)	1,802,581	1,711,311
	4,269,426	4,021,471
Construction in progress	3,758,341	1,316,468
Investments at cost (approximate market quotation 1969—$527,106)	707,327	842,629
Due from current funds	13,401	38,531
	$8,748,495	$6,219,099

Current Assets

	1969	*1968*
Cash	163,471	251,061
Temporary investment in pooled securities	5,728	5,728
Accounts receivable (U. S. Government, 1969—$52,391; 1968—$33,824)	137,757	130,503
Inventories of supplies and bulletins	45,593	47,036
Other assets	6,619	7,146
Due to plant funds	(13,401)	(38,531)
Due to endowment funds	(61,622)	(53,276)
	$ 284,145	$ 349,667

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1969 and 1968

Invested Funds

	1969	*1968*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,575,839	$1,575,720
Endowment funds for awards and scholarships:		
Principal	427,663	427,663
Unexpended income	44,630	39,394
	472,293	467,057
Unrestricted funds functioning as endowment	1,528,190	2,336,190
Retirement fund	217,433	192,543
Pooled investments—accumulated (loss) gain	(53,240)	7,796
	$3,740,515	$4,579,306

Plant Funds

	1969	*1968*
Funds expended for plant, less retirements	9,598,348	6,886,046
Less allowance for depreciation charged thereto	1,802,581	1,711,311
	7,795,767	5,174,735
Accounts payable	240,001	163,204
Unexpended plant funds	712,727	881,160
	$8,748,495	$6,219,099

Current Liabilities and Funds

	1969	*1968*
Accounts payable and accrued expenses	56,419	44,737
Advance subscriptions	30,141	23,712
Unexpended grants—research	33,918	25,727
Unexpended balances of gifts for designated purposes	23,278	23,764
Current fund	140,389	231,727
	$ 284,145	$ 349,667

Note 1—During the current year the Laboratory reduced the estimated useful lives of certain plant assets. This change had the effect of increasing depreciation expense for 1969 by approximately $43,000. Depreciation charges remain, however, from 1% to 5% of the original cost of the assets.

Note 2—The Laboratory has commitments of approximately $2,600,000 for the construction of a teaching laboratory and a dormitory dining-hall.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF OPERATING EXPENDITURES AND INCOME

Years Ended December 31, 1969 and 1968

	1969						
	Salaries and Wages	*Other Costs and Expenses*	*Depreciation*	*Total*	*Charged to Grants*	*Total*	*1968*
Operating Expenditures							
Instruction		$ 14,867	$ 4,885	$ 19,752	$189,107	$ 208,859	$ 217,865
Research		26,824	59,794	86,618	373,786	460,404	505,637
Dormitories	$ 22,893	39,104	29,907	91,904		91,904	69,742
Dining	20,125	41,491	317	61,933		61,933	61,862
Library	32,838	13,729	18,336	64,903		64,903	41,900
Back sets, serials and binding		42,186		42,186		42,186	37,051
Biological Bulletin	4,748	41,252		46,000		46,000	40,219
Support services:							
Apparatus	49,006	44,776		93,782	10,949	104,731	97,698
Supply	85,096	54,611	11,945	151,652		151,652	141,744
Administration	87,150	72,446		159,596		159,596	147,274
Plant operation	136,791	91,808	1,991	230,590		230,590	217,867
Grant expenditures for support services					48,875	48,875	43,584
Other		31,800		31,800		31,800	38,082
	$438,647	$514,894	$127,175	1,080,716	$622,717	1,703,433	1,660,525

MARINE BIOLOGICAL LABORATORY

STATEMENT OF OPERATING EXPENDITURES AND INCOME

Years Ended December 31, 1969 and 1968

Income (continued)						
Instruction	38,110		38,110	189,107	227,217	233,480
Research	123,776		123,776	373,786	497,562	544,646
Dormitories		83,640	83,640		83,640	82,833
Dining		71,938	71,938		71,938	73,135
Library	16,854	27,654	44,508		44,508	46,619
Biological Bulletin		39,789	39,789		39,789	38,451
Support services:						
Apparatus		29,429	29,429	10,949	40,378	31,409
Supply		54,762	54,762		54,762	45,602
Administration		15,336	15,336		15,336	12,769
Investments income		230,225	230,225		230,225	244,364
Gifts used for current expense		34,379	34,379		34,379	13,224
Allowance for indirect costs		74,958	74,958		74,958	96,482
Grants for general support		83,911	83,911		83,911	87,644
Grants for support services				48,875	48,875	43,584
Other		3,658	3,658		3,658	3,212
	$178,740	$749,679	928,419	$622,717	1,551,136	1,597,454
Excess of current expenditures and depreciation over current income			152,297		152,297	63,071
Reduction in plant funds for depreciation			127,175		127,175	86,613
Excess current expenditures			($ 25,122)		($ 25,122)	$ 23,542

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1969

	Balance December 31, 1968	*Gifts and Other Receipts*	*Investment Income*	*Used or Current Expenses*	*Other Expenditures*	*Balance December 31, 1969*
Invested funds.	$4,579,306	$ 31,635 (808,000)(3)	$250,688	$ 229,997	$ 83,117	$3,740,515
Unexpended plant funds	$ 881,160	223,475	24,688		416,596	$ 712,727
Unexpended research grants.	$ 25,727	2,254,014		781,586	1,464,237	$ 33,918
Unexpended gifts for designated purposes	$ 23,764	7,469		7,068	887	$ 23,278
Current fund.	$ 231,727	(7,500)(2) (25,122)(1) 808,000 (3)			866,716	$ 140,389
		$2,483,971	$275,376	$1,018,651	$2,831,553	
Gifts.		230,944				
Grant for facilities construction.		1,464,237				
Grants for research, training and support.		789,777				
Appropriated from current income and other.		24,135				
Net gain on sale of securities.						
(1) Excess of current expenditures over income.		(25,122)				
(2) Gift of boat transferred to plant funds expended. . . .						
(3) Transfer from. . . . . . invested funds.						
		$2,483,971				
Expended for new laboratory and dormitory—dining hall. . . .					2,747,548	
Scholarship awards					8,930	
Payments to pensioners. . . .					13,152	
Loss on sale of securities.					61,036	
Other.					887	
					$2,831,553	

MARINE BIOLOGICAL LABORATORY

Summary of Investments

December 31, 1969

	Cost	*Per Cent of Total*	*Market Quotations*	*Per Cent of Total*	*Investment Income 1969*
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 25,065	2.0	$ 24,325	1.3	$ 1,250
Corporate bonds	711,938	57.4	500,123	28.3	31,158
Preferred stocks	84,771	6.8	69,986	4.0	3,991
Common stocks	419,247	33.8	1,175,447	66.4	42,471
	1,241,021	100.0	1,769,881	100.0	78,870
General educational broad endowment fund:					
U. S. Government securities	53,113	15.9	51,569	12.1	2,650
Other bonds	184,745	55.4	134,487	31.4	8,107
Preferred stocks	15,476	4.6	11,753	2.7	1,244
Common stocks	80,380	24.1	229,913	53.8	5,065
	333,714	100.0	427,722	100.0	17,066
Total securities held by Trustee	$1,574,735		$2,197,603		95,936
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	33,338	5.2	34,000	4.5	1,354
Corporate bonds	134,850	21.3	114,244	15.0	8,274
Preferred stocks	67,040	10.6	56,625	7.4	1,236
Common stocks	398,924	62.9	558,831	73.1	16,400
	634,152	100.0	$ 763,700	100.0	27,264
Less temporary investment of current fund cash	5,728				228
	628,424				27,036
Other investments:					
U. S. Government securities	27,938				1,133
Other bonds	15,029				750
Common stocks	49,634				2,697
Real estate	17,549				
Short-term commercial notes	1,364,000				134,134
	1,474,150				138,714
Total investments of other endowment and unrestricted funds	$2,102,574				165,750
Total					261,686

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS (CONTINUED)

December 31, 1969

	Cost	*Investment Income 1969*
Custodian's fees charged thereto		10,998
Investment income distributed to invested funds		250,688
Plant investments:		
Federal agency and corporate bonds	140,000	7,242
Common stock	564,456	17,394
Preferred stock	2,871	52
	$ 707,327	24,688
Current investments:		
Temporary investment in pooled securities	$ 5,728	228
Total investment income		$275,604

Reference: *Biol. Bull.*, **139**: 80–91. (August, 1970)

THE NUTRITION OF *PARANEMERTES PEREGRINA* (RHYNCHOCOELA: HOPLONEMERTEA). I. STUDIES ON FOOD AND FEEDING BEHAVIOR[1]

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Nemerteans are common organisms in the intertidal zones of temperate regions of the world (Coe, 1943). They live in a wide variety of habitats and can occur in large numbers. Most nemerteans are carnivorous (Coe, 1943), and as abundant predators they may well be important components of the communities in which they live. Since efficient exploitation of available food is a necessary requirement of all animals, studies of feeding and food preference should be of great importance to the field of ecology (Emlen, 1966). Both because of their predatory nature and because they have an interesting means of capturing food, nemerteans are well suited to the study of feeding and food preference.

This paper reports aspects of feeding behavior of *Paranemertes peregrina* Coe, 1901, hereafter referred to as *Paranemertes*, a predatory nemertean which occurs along the entire Pacific coast of North America from the Aleutian Islands in Alaska to Ensenada, Mexico (Coe, 1940) in both rocky and muddy bay intertidal habitats. The feeding habits of *Paranemertes* have previously received little attention, being only briefly mentioned by Coe (1905), MacGinitie and MacGinitie (1949), and Correa (1964). The MacGinities (1949, and personal communication) reported that *Paranemertes* feeds at low tides on nereid polychaetes.

Specifically, this report concerns (1) a description of the feeding process of *Paranemertes*, including steps in prey capture; (2) a comparison of *Paranemertes* from exposed and sheltered rocky intertidal and muddy bay areas for food in nature and food preferences in laboratory experiments; and (3) a description of an escape response of nereid prey to *Paranemertes*. This study is the beginning of an ecological study now in progress on the role of *Paranemertes* as a predator in these types of communities.

MATERIALS AND METHODS

Four study areas were selected where *Paranemertes* is fairly abundant. These were: (1) English Camp at Garrison Bay, San Juan Island, Washington, a protected muddy bay area; (2) the ferry landing at Edmonds, Washington, a concrete and metal structure with large boulders piled against it; (3) boulders on the sandy beach to the north of the ferry landing at Edmonds; and (4) the rocky outcropping at Mukkaw Bay, south of Cape Flattery, open Pacific coast,

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[1] Part I of this paper is based on a thesis submitted by P. Roe in partial fulfillment of the requirements for a Master of Science degree, University of Washington, 1967.

Washington. Garrison Bay and the two rocky areas at Edmonds are in protected waters; Mukkaw Bay is exposed to strong wave action. *Paranemertes* was found on the mud at Garrison Bay, and among and below the barnacles and mussels on the rocks in the other areas.

Field observations and collections of over 500 *Paranemertes* were made at the four study areas during low tides from October 1966 through April 1967, monthly at Garrison and Mukkaw Bays and nearly weekly at Edmonds. Specimens of *Paranemertes* were put singly into 2-oz jars partly filled with clean cooled sea water. All specimens were kept near 10° C.

Polychaetes from each study area were collected to determine what species lived in association with *Paranemertes* and to be used in food preference experiments. Polychaetes were identified from Hartman, 1968 and Berkeley and Berkeley 1948, 1952. Potential prey from other phyla which occurred in these habitats were not used for food preference experiments since initial examinations of *Paranemertes* faeces and initial preference experiments gave positive results only for polychaetes.

Since each nemertean was kept in a separate container, its faeces from food eaten in nature could be collected from the water in which it was kept. *Paranemertes* faeces consist of bundles of polychaete setae, facilitating identification of the prey. Faeces and worms that had fed in nature were preserved in 70% alcohol.

In food preference experiments a potential prey was put into a container with a nemertean which had been kept without food for one week, and the reactions of the nemertean and prey were observed. Reactions of the nemerteans were graded in three categories, after contact was made with the prey. Step 1, absence of reaction or jerking back of the head was considered a negative response. Step 2, eversion of the proboscis, and Step 3, ingestion of prey, were considered positive responses. If a negative response occurred following presentation of a polychaete other than a nereid, a nereid was offered subsequently, since preliminary work indicated that *Paranemertes* has a strong preference for nereids. The experiment was excluded from analysis of results if there was a negative response to the nereid control. Preliminary data suggested that *Paranemertes* has a slight "memory," *i.e.*, a nemertean offered a nereid followed soon by a different food responds positively at first to the second food. This "memory" apparently does not last more than a few minutes. Therefore, if a given *Paranemertes* was used for more than one feeding experiment, it was returned to its container after each experiment and not used again for at least one hour. When possible, each prey type was presented in ten trials. The length of time before defecation was recorded in several instances. Fisher's exact test (Siegel, 1956) was used except where otherwise indicated to examine the significance of food preference results.

An escape response of nereid prey to *Paranemertes* was observed in nature and in *Paranemertes* food preference tests. Experiments were run to determine (1) if nereids responded more vigorously to *Paranemertes* than to simple tactile stimulus and (2) what parts of the nereid body are most sensitive to *Paranemertes*.

OBSERVATIONS AND RESULTS

General observations in nature

Paranemertes is an active predator during low tide. Worms were observed to emerge from crevices and from the mud as the tide receded and they were observed feeding out of water at low tide. They are not merely stranded on the rocks as the tide goes out.

Few *Paranemertes* come out when it is raining, even during low tides. During inclement weather aggregations of *Paranemertes* can be found in cracks or sheltered crevices in rocky areas. *Paranemertes* crawls out into the open more often at night or on cloudy days than it does on sunny days.

Feeding process

Feeding of *Paranemertes* has been analyzed as a 3-step process. Step 1, prey contact: Contact of the anterior edge of *Paranemertes'* head with a prey organism and recoil of *Paranemertes'* anterior end is the first step of the feeding process. This recoil almost always occurs, even if the prey proves an unacceptable species. Recoil is often accompanied by a 90° or a 180° turn of the head away from the prey and a crawling away if the prey is not acceptable. The initial head withdrawal is more vigorous if the prey is a nereid.

Preliminary tests and observations suggested that *Paranemertes* has little or no distance chemoreception for use in finding prey. Nereids had to come into contact with the anterior rim of a *Paranemertes'* head before the nemertean responded. Water from jars in which nereids lived caused no response when pipetted into a jar with a *Paranemertes*. In two of eleven tries, *Paranemertes* responded by slight eversion of the proboscis to the tube of a recently-removed nereid. Once a *Paranemertes* followed the fresh mucus trail of a nereid. Contact with nereids or their immediate and fresh products appears to be a requirement for recognition and elicitation of the feeding response by *Paranemertes*.

Step 2, prey capture: The second step in the feeding process is eversion of the proboscis, often rapidly and with great force. Prey much stronger than the nemertean may be paralyzed within a few seconds by this action. In the laboratory, eversion of the proboscis occurs almost every time a nereid is presented, but only rarely in response to other prey organisms (Tables I–IV). For this reason Step 2 is considered a positive feeding response. In the laboratory *Paranemertes* often paralyzes a nereid, then does not eat it.

The proboscis everts to a given point, stops, then moves again, either inverting or everting more, then stops again. These stops are sometimes accompanied by a visible jabbing motion. The stylet of *Paranemertes* is small (Coe, 1905) and difficult to follow during rapid proboscis movements. However, it was observed moving forward to the point where the end was in contact with the prey. It is believed that the stylet venom is pumped in the prey during these stops. Paralysis is complete only in the part of the prey around which the proboscis has been wound, and often a nemertean must envelop the prey 3 or 4 times in different places before the effect is complete. The proboscis can be everted a short or long distance and the stylet can be terminal in either case. When the nemertean is through puncturing the prey in one place, it can withdraw the stylet, evert the end of the proboscis still more, and stab in a new and more distant place on the prey. The initial force for eversion is hydrostatic (Bohmig, cited by Clark, 1964), but further, more deliberate eversion appears to be controlled by the proboscis musculature. Either the central stylet is replaced with great rapidity or *Paranemertes* does not lose its stylet readily. Proboscides cut off while wrapped around a prey or shortly thereafter always showed the central stylet intact and always showed accessory stylets in the accessory sacs. The proboscis often winds tightly around the prey, and combined effects of prey movement, squeezing, and stylet action frequently cause the prey to tear apart. However, if the prey is not badly damaged, or if it is fairly large, the effect of the venom wears off in about 20 minutes. The venom paralyzes but does not kill the prey.

The proboscis is lined with conical gland cells that secrete a sticky mucus-like substance upon contact with a prey, enveloping the prey where the proboscis was wrapped around it. This sticky substance functions to hold the prey and may have some paralytic function as well.

Step 3, ingestion: After a prey is paralyzed *Paranemertes* withdraws the proboscis, losing contact with the prey for a few seconds. It then moves over the prey, feeling for a place to start the sucking-ingestion process, the third of the feeding stages. *Paranemertes* will start sucking any place on the prey which it can surround with its distended mouth. Prey can be ingested tail or head first, or be bent into a v- or j-shape and ingested from the side. Muscular peristalses from the posterior tip anteriorad often accompany ingestion. Most of ingestion is accomplished by the musculature around the mouth. There are sometimes posterior-to-anterior peristaltic waves of the region just posterior to the mouth. The lips also expand lengthwise, then close over a new portion of the prey, then contract, drawing in the prey. Sometimes small prey seem to be glided in as if by ciliary action. If a prey begins to move during ingestion, or if the prey is large and the *Paranemertes* is having difficulty ingesting it, the proboscis is often everted even while the anterior end is highly distended. When prey diameter is less than the distended mouth, ingestion only takes a few minutes; the entire feeding process can take place in 2 to 3 minutes. The limiting factor in ingestion is prey diameter. *Paranemertes* cannot ingest a prey whose diameter is far greater than its own. Prey length makes little difference. One *Paranemertes* was observed eating a nereid longer than itself.

Slight variations in the feeding process occur when syllids or spionids are eaten. The greatest differences were observed during feeding on *Syllis*. Even though the syllid hardly moved, the nemertean everted its proboscis more than 10 times, yet the syllid was hardly affected, suggesting that syllids might be partially refractory to the venom. A posterior piece of the syllid was broken off, and the proboscis continued to entwine the piece. Ingestion took several minutes even though the syllid was thin and only about an inch long, and the piece being ingested even smaller. Eating consisted of tearing a piece off, wrapping the proboscis around that piece, ingestion, then repeating the process on another piece further anterior.

On a few occasions, when a *Paranemertes* was offered a nereid that had been previously paralyzed, step two was skipped.

Length of time before defecation

Observations of 12 specimens of *Paranemertes* every 2 hours after laboratory feedings showed that defecation occurs from 12 to more than 33 hours after feeding. Observations at longer intervals from field collections and laboratory feedings gave the same results.

Observations of feedings in nature

At Garrison Bay 44 of 246 specimens of *Paranemertes* collected (18%) were observed feeding or defecated shortly after being taken to the laboratory. Of those that had fed in nature, 36 (81.8%) had eaten *Platynereis bicanaliculata*, 7 (15.9%) had eaten other nereids (setae of nereids other than *Platynereis* were not identifiable to species), and only 1 (2.3%) had fed on a non-nereid polychaete. A total of 97.7% had eaten nereid prey.

At Mukkaw Bay 8 of 42 worms collected (19%) had recently fed, all on nereid prey, and probably all on *Nereis vexillosa* as that was the only nereid species found in that habitat.

Twenty of 56 worms (35%) collected at Edmonds beach had recently eaten. Seven of these were observed feeding. Food at this habitat consisted of 11 nereids (probably *N. vexillosa*) (55%), 8 *Polydora* sp. (40%), and 1 *Syllis* sp. (5%). Although the majority of worms from Edmonds beach had fed in nature on nereids, the nemerteans from this location had a more varied diet than did those at Garrison or Mukkaw Bays.

At the Edmonds ferry landing 33 of 140 worms collected (23.5%) had recently fed. Six of these were observed while feeding. The diet at the ferry landing consisted of 22 (67.7%) *Syllis* sp., 8 (24.2%) *Polydora* sp. and 3 (9.1%) *Lepidonotus squamata*. No nereid faeces were collected from *Paranemertes* from the ferry landing. Nereids were quite rare in numerous polychaete samples from the ferry landing rocks, and were probably too scarce to constitute much of the diet of *Paranemertes*.

Food preference experiments

Tables I–IV show possible prey species tested with *Paranemertes* from each study area. In most cases prey species found in a given study area were tested with *Paranemertes* from the same area. However, *Platynereis* was tested with *Paranemertes* from all study areas even though it was found only at Garrison Bay. Members of all macroscopic polychaete families collected from each area were tested. One *Neanthes brandti* (Nereidae) was found at Garrison Bay, and individuals of *Paranemertes* have been observed attacking *Neanthes*. However, adults are far too large for the nemerteans to be able to ingest; they were therefore not considered in laboratory experiments.

In the tables, steps 1, 2 and 3 refer to feeding reactions of *Paranemertes* to the prey. Step 1 is considered a negative response, steps 2 and 3 are considered positive responses. The column "total positive trials" sums all trials in which a *Paranemertes* responded positively either to a test prey or to a control nereid. The column "negative responses to controls" refers to the number of trials in

TABLE I

Feeding experiments with Garrison Bay Paranemertes

Food	Steps in feeding response			Total positive trials	Negative responses to nereid controls
	Initial Step 1	Final			
		Step 2	Step 3		
Lumbrineris inflata	5			5	4
Ophiodromus pugettensis	10			10	0
Nainereis laevigata	11			11	2
Glycinde sp.	6			6	3
Dorvillea sp.	8			8	1
Cirratulid	8	1		9	1
Terebellid	5			5	5
Phyllodocid	8			8	2
Polydora sp.	7	1		8	2
Lepidonotus squamatus	6		1	7	0
Armandia brevis	9		1	10	1
Nereis vexillosa	1	5	5	11	—
Platynereis bicanaliculata	10*		17	27	—
Totals	94	7	24	125	21

* These worms were fed by invertebrate zoology students. I do not know if the worms responded at Step 1 or Step 2. These data are conservatively listed as negative responses.

which a *Paranemertes* did not respond to either test or control prey. There were no controls when nereids were the test prey.

In the laboratory specimens of *Paranemertes* from Garrison Bay show a very strong preference for nereids (Table I). In only one case was any other polychaete ingested, and in only 4 of 87 tries did a *Paranemertes* react positively to other possible prey organisms. The nemerteans from Garrison Bay did not prefer one nereid species to the other (χ^2 test with Yates' correction: $P > 0.5$).

Although there are few experiments with specimens of *Paranemertes* from Mukkaw Bay, results show (Table II) a significant difference in response by

TABLE II

Feeding experiments with Mukkaw Bay Paranemertes

Food	Steps in feeding response			Total number of trials
	Initial Step 1	Final		
		Step 2	Step 3	
Phyllodocid	5			5
Syllis sp.	8			8
Nereis vexillosa		10	1	11
Platynereis bicanaliculata	1	10	1	12
Totals	14	20	2	36

TABLE III

Feeding experiments with Edmonds Beach Paranemertes

Food	Steps in feeding response			Total positive trials	Negative responses to controls
	Initial	Final			
	Step 1	Step 2	Step 3		
Hemipodus borealis	7			7	0
Polydora sp.	9			9	2
Syllis sp.	8	1		9	2
Eteone sp.	10			10	2
Lepidonotus squamatus	7	1		8	1
Nereis vexillosa	1	5	1	7	—
Platynereis bicanaliculata	2	3	4	9	—
Totals	44	10	5	59	7

Pairs with Significant Difference at the 5% Level (Fisher's Exact Test)

Nereis—Syllis	$P = 0.01$
Nereis—Polydora	$P < 0.005$
Nereis—Lepidonotus	$0.025 > P > 0.01$
Nereis—Eteone	$P < 0.005$
Nereis—Hemipodus	$0.01 > P > 0.005$
Platynereis—Lepidonotus	$P = 0.025$
Platynereis—Polydora	$P = 0.005$

TABLE IV

Feeding experiments with Paranemertes *from Edmonds Landing*

Food	Steps in feeding response			Total positive trials	Negative responses to controls
	Initial	Final			
	Step 1	Step 2	Step 3		
Syllis sp.	9	4	2	15	2
Polydora sp.	7	3	3	13	0
Lepidonotus squamatus	10	1		11	1
Hemipodus borealis	8	1		9	0
Eteone sp.	8			8	0
Nereis vexillosa		6	4	10	—
Platynereis bicanaliculata		3	5	8	—
Totals	42	18	14	74	3

Pairs with Significant Difference at the 5% Level (Fisher's Exact Test)

Platynereis—Syllis	$0.025 > P > 0.01$
Platynereis—Polydora	$0.05 > P > 0.025$
Platynereis—Lepidonotus	$P < 0.005$
Platynereis—Hemipodus	$P < 0.005$
Platynereis—Eteone	$P < 0.005$
Syllis—Eteone	$P = 0.05$
Polydora—Eteone	$P = 0.05$

Paranemertes to *Nereis* or *Platynereis* and syllids ($P < 0.005$) and to *Nereis* ($0.01 > P > 0.005$) or *Platynereis* ($P = 0.005$) and phyllodocids. There was no significant difference in response to the nereid species offered. It can be concluded that Mukkaw Bay individuals also prefer nereid over non-nereid food.

There was a significant difference in the response of specimens of *Paranemertes* from Edmonds beach to *Nereis* or *Platynereis* and to non-nereid foods. There was no significant difference in responses among the non-nereid species, and there was no significant difference in response between the nereid species offered (Table III). It can be concluded that Edmonds beach individuals also prefer nereid over non-nereid food and have no preference among the nereids.

Specimens of *Paranemertes* from Edmonds ferry landing again showed preference of nereids to all other prey, and there was no significant difference in the response to the two nereid species offered. The nemerteans reacted positively to *Syllis* and *Polydora*, and even ingested them, and there was a significant difference at the 5% level in the response of *Paranemertes* to these two species and *Eteone*, to which *Paranemertes* showed total lack of response.

Differences between nereids

Although specimens of *Paranemertes* showed no significant preference of one nereid over another, there was a significantly greater number of successful feedings during the food preference tests when *Platynereis* was the prey than when *Nereis vexillosa* was the prey. Several times *Paranemertes* could not find a place on *Nereis* from which to start the sucking ingestion process (step 3 of the feeding process). If trials in which step 3 was started, but in which *Paranemertes* derived no nourishment from the prey, are combined with step 2 as unsuccessful positive trials (Table V), *Paranemertes* was significantly more successful in feeding on *Platynereis* than on *Nereis vexillosa* ($\chi^2 = 5.957, 0.02 > P > 0.01$).

Nereis vexillosa appears to be a more aggressive animal than *Platynereis*. On several occasions during feeding experiments a *Nereis* bit a *Paranemertes'* proboscis when the proboscis was wrapping around it. The *Nereis* sometimes

TABLE V

Unsuccessful positive responses versus *completed feedings by* Paranemertes *to* Platynereis *and* Nereis

Source of experimental animals	Food	Unsuccessful positive responses	Complete feedings
Garrison Bay	*Nereis vexillosa*	6	4
	Platynereis bicanaliculata	10	17
Mukkaw Bay	*Nereis vexillosa*	10	1
	Platynereis bicanaliculata	11	0
Edmonds Beach	*Nereis vexillosa*	5	1
	Platynereis bicanaliculata	4	3
Edmonds Landing	*Nereis vexillosa*	9	1
	Platynereis bicanaliculata	4	4
Totals	*Nereis vexillosa*	30	7
	Platynereis bicanaliculata	29	24

did not let go for more than an hour, making the feeding process very drawn out. On one occasion a *Nereis* bit the tail off a *Paranemertes.* These actions were never observed when *Platynereis* was the prey. Small *Nereis* were used for feeding experiments; however, *Nereis* as small as some *Platynereis* used were difficult to find. Size difference might account for part of the increased difficulty *Paranemertes* had with *Nereis.*

Nereid escape response

It was observed, both in nature and in the laboratory food preference tests for *Paranemertes,* that nereid polychaetes have an escape response from *Paranemertes.* Nereids swim rapidly away upon contact with *Paranemertes* if water is present. If they cannot swim, as is often the case during low tides in nature when *Paranemertes* is feeding, they jerk away and crawl off rapidly. Responses of nereids in tests to determine if nereids responded to *Paranemertes* more strongly than to simple tactile stimulus and to determine their more sensitive parts were categorized as five levels (Table VI). Data from *Nereis vexillosa* and *Platynereis* were similar and are lumped. Response to tactile stimulus was determined only for a sensitive part of the nereids, the posterior cirri.

TABLE VI

Responses of nereids to Paranemertes *and to tactile stimulus*

Part of nereid touched	Response to *Paranemertes*				
	Degree of response				
	0	1	2	3	4
	Number of times response reached				
Palps or anterior end	2	0	4	0	1
Parapodia	0	0	1	0	2
Dorsum or venter	0	0	1	0	4
Posterior cirri	0	0	6	3	5
	Response to tactile stimulus				
Posterior cirri	4	2	1	0	0

Degree of response by nereids to *Paranemertes* or to tactile stimulus
0 = neutral
1 = slight increase in crawling rate
2 = jerk and rapid crawling
3 = flip of tail and rapid crawling
4 = swimming

If response levels 0 and 1 are considered negative and levels 2, 3, and 4 are considered positive responses, there is a significant difference ($\chi^2 = 15.90$, $P < 0.001$) between nereid response to *Paranemertes* and to tactile stimulus from a pencil or probe. Fisher's exact test shows no significant difference in response from any one of the four body parts tested. The difference between response to tactile stimulus and response to *Paranemertes* of nereid palps was not significant at the 5% level, but the difference was significant for the other three areas tested (parapodia—tactile stimulus, $P = 0.05$; dorsum—tactile stimulus, $0.025 > P > 0.01$; posterior cirri—tactile stimulus, $P < 0.005$).

Although very few trials were made, nereids did not respond with the swimming escape response when they came into contact with *Amphiporus formidabilis*, *Emplectonema gracile*, or *Zygonemertes virescens*, other nemerteans which occur in the same habitats with *Paranemertes* and the nereids, but which to my knowledge, do not eat nereids.

Discussion

The feeding process of *Paranemertes* differs in only minor details from feeding reported for other vermivorous nemerteans. For example, heteronemerteans as *Lineus* do not lose proboscis contact with the prey while searching for a place to start ingestion (Beklemishev, 1955; Jennings, 1960); *Paranemertes* can afford such behavior since it paralyzes its prey. *Paranemertes*, like *Cephalothrix* species (Jennings and Gibson, 1969), cannot ingest polychaetes that are much greater in diameter than it is. *Lineus* can ingest prey several times its diameter (Gontcharoff, 1948; Beklemishev, 1955). *Paranemertes* has no distance chemoreception for finding food; *Prostoma* (Reisinger, 1926), *Cerebratulus* (Wilson, 1900; Coe, 1943), and two species of *Lineus* (Jennings and Gibson, 1969) all find prey by long range chemoreception.

A major difference between feeding of *Paranemertes* and most other nemerteans is the high degree of specificity in food preference in *Paranemertes*. *Amphiporus lactifloreus* was reported to have narrow preference for *Gammarus locusta* in laboratory feedings (Jennings and Gibson, 1969), but most nemerteans studied so far eat a wide variety of prey (Roe, 1967; Gibson, 1968, 1970). The specialized preference of *Paranemertes* for nereids in the laboratory is reflected in nature by the high percentage of nereid food in the diet in areas where this preferred food is available. Although *Paranemertes* at the Edmonds ferry landing did not eat nereids because they were unavailable, these same nemerteans showed strong preference for nereid food in the laboratory experiments. These *Paranemertes* are quite small, even as mature adults, compard to the sizes of Garrison and Mukkaw Bay *Paranemertes*. Their small size is possibly an effect of the lack of preferred food in their diet. *Paranemertes* may be able to capture nereids with much less energy expended per unit energy gained than they can with other prey, as evidenced by the difficulty of feeding by *Paranemertes* on syllids. *Paranemertes* seems to have an optimal feeding strategy by specializing on nereids when they are available and taking a wider range of prey when nereids are uncommon. A maximum of selectivity in food types when food is common, and a decrease in selectivity when food is scarce is the optimal feeding strategy for predators (Emlen, 1966).

The absence of distance chemoreception in *Paranemertes*, combined with its highly specialized predation behavior and diet, plus the well-developed escape response of nereids to it suggest a pattern of evolution in environments where preferred food is so common that distance chemoreception is unnecessary.

I wish to thank Dr. Alan J. Kohn for his assistance throughout this work. I would also like to thank Dr. Robert T. Paine for letting me go with him to Mukkaw Bay every month, and Dr. Robert Fernald for providing facilities at Friday Harbor Laboratories for my use.

SUMMARY

1. *Paranemertes peregrina,* an abundant hoplonemertean in rocky and muddy intertidal habitats of Washington, feeds on polychaetes during low tide periods.

2. Feeding by *Paranemertes* involves three steps. The first stage is a recoil of the *Paranemertes'* head upon contact with a prey. Contact must be made with the prey; *Paranemertes* does not find prey by distance chemoreception. The proboscis is everted and wraps around the prey in step two. The prey is temporarily paralyzed or is killed. In step three the prey is ingested by means of sucking motions from muscles around the mouth. Defecation occurs from 12 to 33 hours after feeding.

3. Specimens of *Paranemertes* ate mainly nereid polychaetes at the three study areas where nereids were available. At the fourth study area the nemerteans ate a wider variety of polychaetes. Experiments to test food preference showed that specimens of *Paranemertes* from all study areas strongly preferred nereids to members of other polychaete families.

4. Nereid polychaetes have a swimming escape response from *Paranemertes.*

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THE NUTRITION OF *PARANEMERTES PEREGRINA* (RHYNCHOCOELA: HOPLONEMERTEA). II. OBSERVATIONS ON THE STRUCTURE OF THE GUT AND PROBOSCIS, SITE AND SEQUENCE OF DIGESTION, AND FOOD RESERVES[1]

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The division of the Rhynchocoela into Anopla and Enopla is partly based upon the morphology of the gut and proboscis (Hyman, 1951). In anoplan nemerteans the mouth and proboscis pore are quite separate and the proboscis lacks stylet armature, whereas enoplans have the gut and proboscis opening anteriorly via a common rhynchodaeal aperture. In addition, the hoplonemertean enoplans have their proboscis armed by a characteristic stylet apparatus.

Despite these major differences, the basic digestive physiology of both groups is similar. It consists of an acidic extracellular proteolytic phase, followed by the phagocytosis of food particles and their subsequent intracellular digestion by means of proteases, carbohydrases and lipases acting in harmony (Jennings and Gibson, 1969). Intracellular digestion occurs in two stages, first acidic and secondly alkaline, with acid and alkaline phosphatases being associated with the respective phases.

An exception to this general rule is found in the atypical microphagous bdellonemerteans, where the loss of the carnivorous habit has resulted in a reduction of the emphasis placed upon proteolysis concurrent with an increase in the amount of carbohydrase activity at both the extra- and intracellular sites (Gibson and Jennings, 1969).

In all the hoplonemertean species so far investigated the endopeptidases responsible for extracellular proteolysis are produced and secreted by the gastrodermal columnar cells. Functional gland cells are present in the intestinal epithelium, but their precise role in the digestive processes has not yet been determined.

Greater variation in hoplonemerteans is, however, found in the acid-secreting mechanisms of the foregut, and two distinct types can be recognized (Jennings and Gibson, 1969). *Prostoma rubrum*, in common with anoplan species, possesses acidophilic gland cells which at all times exhibit demonstrable carbonic anhydrase activity, but amphiporids and tetrastemmids lack this enzyme completely although still producing acidic secretions. These variations in physiology may be attributed to differences in feeding, as *Amphiporus*, and probably *Tetrastemma* also, possesses a specialized feeding mechanism, in contrast to *Prostoma* which

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feeds in the manner more typical of the phylum as a whole (various authors, summarized by Jennings and Gibson, 1969).

The food reserves of nemerteans consist principally of fat globules stored in the gastrodermis, although some deposits on occasion can also be found in the general body parenchyma. No protein reserves have been recorded for the group, and the storage of glycogen appears to be of secondary importance. Glycogen occurs as tiny granules scattered throughout the gastrodermis, parenchyma and musculature, with occasional aggregations around the gonads (Reisinger, 1926; Jennings, 1960; Gibson and Jennings, 1969; Jennings and Gibson, 1969).

Until recently the common Pacific hoplonemertean *Paranemertes peregrina* Coe had been little investigated with respect to its nutrition. Roe (1967, 1970) has reported on the food and feeding behavior of this species, and the present study forms a logical sequence to this investigation as well as adding to our knowledge of the digestive physiology in nemerteans.

MATERIALS AND METHODS

Specimens of *Paranemertes peregrina* were obtained from intertidal muddy shores at Garrison Bay and Snug Harbor, San Juan Island, Washington, during July and August 1969.

Histological studies on the structure of the gut and proboscis were made on specimens fixed in marine Bouin, Susa, or 10% neutral formalin containing 3% sodium chloride. Paraffin wax (56° C m.p.) sections cut at 6–8 μ were subsequently stained by routine methods, including hematoxylin and eosin, Feulgen, Mayer's hemalum, periodic acid-Schiff (PAS), Mallory's trichrome, 1% aqueous Alcian blue (for mucopolysaccharides), or by the bromphenol blue technique of Johri and Smith (1956) for proteins.

The site and sequence of digestion was determined from the examination of specimens fixed at progressive time intervals following an observed meal. In all cases the readily accepted nereid polychaete *Platynereis bicanaliculata*, collected from the same areas as *Paranemertes*, was used as the food.

Specimens were fixed for 2–4 h at 4° C in 10% formalin containing 3% sodium chloride, phosphate-buffered to pH 7.0. Following fixation they were either washed in ice-cold distilled water and sectioned directly on an International Harris Model CTD cryostat, or dehydrated through graded acetones at 4° C, cleared in xylene at 18-22° C, and infiltrated *in vacuo* in paraffin wax of melting point 45° C. Paraffin sections were mounted on albumenized slides, air dried at room temperature, and dewaxed before rinsing in absolute acetone prior to incubation for enzyme visualization. Cryostat sections were similarly subjected to acetone treatment in order to remove fats before being incubated. All dehydration, clearing and infiltration times were kept to a minimum suitable to the size of the specimens being processed.

The following methods were used in the investigation of enzymes present: the Hausler (1958) method for carbonic anhydrase; the Hess and Pearse (1958) indoxyl acetate method for cathepsin-C type endopeptidases, as used by Jennings (1962a, 1962b), Rosenbaum and Ditzion (1963), Jennings and Mettrick (1968), Jennings and Gibson (1969), and Jennings and Gelder (1969); the Burstone and Folk (1956) L-leucyl-β-naphthylamide technique for exopeptidases of the leucine-aminopeptidase type; the indoxyl acetate (Holt, 1958) and α-naphthyl acetate (Gomori, 1952) methods for non-specific esterases; the Gomori (1952) Tween 80 method for lipase; the Burstone (1958) azo-dye technique for acid phosphatase; and calcium salt method (Gomori, 1939) for alkaline phosphatase.

Controls used for these histochemical methods included heat-inactivated sections and media from which the specific substrate had been omitted.

The distribution of the food reserves was studied in paraffin sections of specimens fixed either in Flemming's osmium tetroxide fluid (for fats), or 90% alcohol containing 1% picric acid and subsequently stained by the PAS or Bauer methods (for glycogen).

OBSERVATIONS

Structure of the gut and proboscis

As in other monostyliferous hoplonemerteans, the gut and proboscis of *Paranemertes* lack separate external openings. A single anterior pore, the rhynchodaeal aperture, opens from the anterior tip into a somewhat cone-shaped chamber, the rhynchodaeum, and it is from the back of this that the proboscis and gut open dorsally and ventrally respectively (Fig. 1).

The rhynchodaeal epithelium possesses no gland cells and consists only of ciliated cuboidal cells 6–9 μ tall overlying a thin basophilic basement membrane. In common with the remainder of the gut, the rhynchodeum has no specific musculature associated with it, although numerous obliquely arranged fibers are embedded in the surrounding parenchyma. In most specimens local aggregation of muscle fibers at the junction of proboscis and rhynchodeum is suggestive of a possible sphincter.

Around the rhynchodaeum can also be found the lobular frontal glands, which extend posteriorly to just behind the cerebral ganglia, and the paired cerebral organs, which open anteriorly into the cephalic slits.

The gut is divisible histologically into two distinct regions, the foregut and the intestine. Unlike the situation reported for many other hoplonemertean species, *Paranemertes* does not possess either a distinct esophagus or a pyloric tube, and the foregut opens directly into the rhynchodeum and intestine at the appropriate points. The intestine extends ventrally and anteriorly as a blind-ending cecum, both intestine and cecum bearing numerous, often long, multilobed diverticula.

The foregut epithelium is folded and glandular, and consists of two distinct cell types. The principal components are columnar cells 45–60 μ tall and 6–8 μ wide bearing dense distal cilia 4–5 μ long. These cells are filled with a coarsely granular basophilic cytoplasm that fails to react to histochemical stains for either mucus or protein. Their single oval or spherical nucleus, 3–4.5 μ in diameter, is situated proximally. Evidence of cytoplasmic vacuolation can frequently be found, particularly in recently fed specimens.

Between the basophilic cells are non-ciliated pyriform gland cells of similar height but only 4–6 μ width, packed with acidophilic proteinaceous spheres of 1 μ or less diameter. Gland cell nuclei, 4–5 μ long and 2–2.5 μ wide, are positioned proximally with their long axes approximately at right angles to the epithelial basement membrane.

Both cell types are secretory when fully developed. The basophils extrude their contents to the foregut lumen between their distal cilia, but the acidophils open either directly to the lumen or discharge their spheres via secretory tracts extending between the columnar cells. Shortly after ingestion is commenced, evidence of secretion can be found in the foregut lumen, the basophilic component appearing as finely particulate and irregular "strings," the acidophilic spheres complete and isolated. There is some evidence to suggest that the acidophilic secretions initially expand after discharge, and then rupture to release their contents into the lumen.

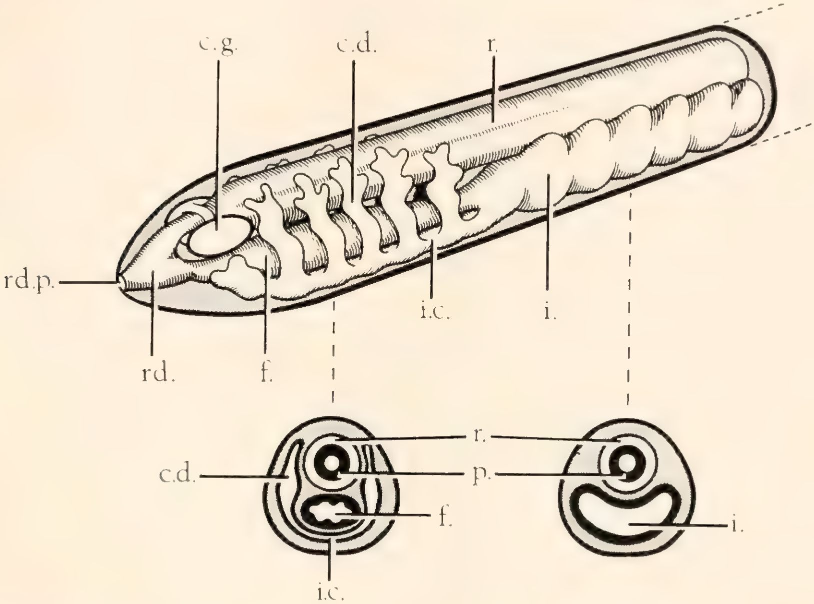


FIGURE 1. *Paranemertes peregrina.* Stereogrammatic representation to show the relative positions of the proboscis and alimentary canal, characteristic of this type of hoplonemertean. The appearance of the body in transverse section is shown for two points in the lower diagrams; c.d., cecal diverticulum; c.g., cerebral ganglion; f., foregut; i., intestine; i.c., intestinal cecum; p., proboscis; r., rhynchocoel; rd., rhynchodeum; rd.p., rhynchodeal pore.

The distribution of the adipophilic glands in the foregut is such that they are concentrated in the anterior half where they and the columnar cells occur in approximately equal numbers. A short region immediately adjoining the rhynchodeum tends to lack these glands, however, and it is probable that this portion of the foregut is equivalent in the esophagus described for other hoplonemertean species. The posterior foregut epithelium shows a progressive decrease in the density of the acidophils as the intestine is approached, and in the region just anterior to the hindgut the ratio of gland to columnar cells is 1:20 or more.

A distinct pyloric tube is absent from *Paranemertes* and there is no decrease in epithelial height although the foregut tends to be less folded. At the junction of foregut and intestine a loose aggregation of circular and oblique muscle fibers is found in the surrounding parenchyma. These may serve as sphincter muscles to this part of the gut, although such an arrangement was not seen in all specimens examined.

The intestinal wall, or gastrodermis, is in its structure very similar to that described for other species. It consists of acidophilic pyriform gland cells interspersed between ciliated columnar cells. In starved specimens the columnar cells are 60–80 μ tall and 6–8 μ wide, their sparsely distributed distal cilia extending 12–14 μ into the intestinal lumen. Subspherical nuclei 2–2.5 μ in diameter are embedded proximally in the cytoplasm, the latter being finely particulate and possessing no particular staining affinities. The proximal regions of the columnar cells also contain variable numbers of acidophilic proteinaceous inclusions up to 3–4 μ diameter, and these react positively to the Hess and Pearse technique for endopeptidases.

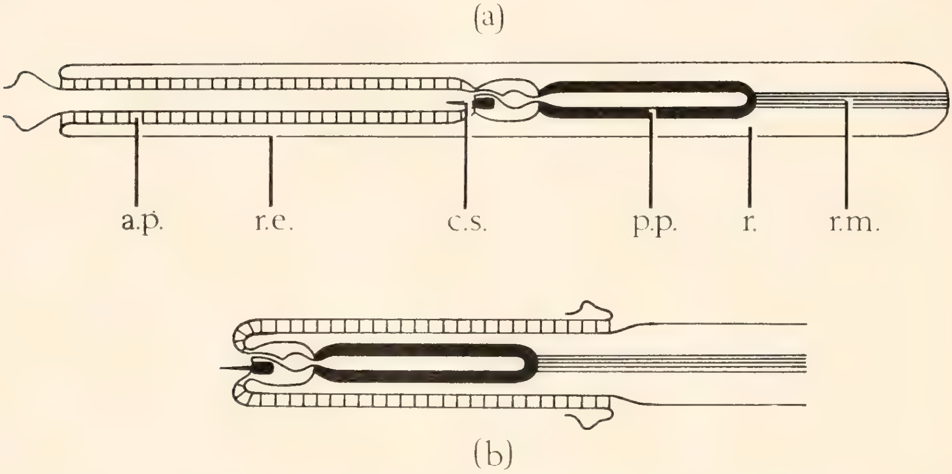


FIGURE 2. *Paranemertes peregrina.* Diagram to show the relationships between the parts of the proboscis in the retracted (a) and protruded (b) positions. Note how in (b) the central stylet is terminal; a.p., anterior proboscis epithelium; c.s., central stylet positioned in muscular bulb; p.p., posterior proboscis epithelium; r., rhynchocoel; r.e., rhynchocoel endothelium; r.m., proboscis retractor muscle.

In contrast, the narrower gland cells, filled with acidophilic proteinaceous spheres of maximum diameter 1.5 μ, fail to react to histochemical methods for either endopeptidases or non-specific esterases, although their contents are discharged into the intestinal lumen and clearly play some part in the extracellular digestive processes.

Gland cells are most numerous in the anterior intestine and cecum, their numbers decreasing posteriorly so that they are almost absent from the region near the anus. The ratios of gland to columnar cells are about 1:1 and 1:30, respectively.

The proboscis of *Paranemertes* is armed by a single, needle-shaped, central stylet, and lies coiled in a rhynchocoel which extends for only about one-quarter of this body length. The rhynchocoel is lined by a thin endothelium overlying muscle layers comprised of inner longitudinal and outer circular fibers.

Three distinct regions of the proboscis can be recognized. In the retracted position (Fig. 2a) these are an anterior thick-walled tube, a short central muscular bulb housing the stylet apparatus, and a posterior acidophilic portion whose rearmost extremity is connected to the rhynchocoel by the proboscis retractor muscle.

The anterior proboscis epithelium is composed of two cell types arranged in a distinctive manner (Fig. 3). Elongate pyriform gland cells packed with proteinaceous acidophilic spheres of less than 1 μ diameter are interspersed with irregular-shaped columnar cells whose distal regions are filled with finely particulate, nonproteinaceous basophilic cytoplasm. In the retracted position the cellular arrangement is somewhat obscured (Fig. 4), but when the proboscis is protruded the epithelium forms definite papillae, each papilla consisting of a series of groups of acidophilic cells flanked by columnal cells. The overall epithelial height at this time is approximately 40–50 μ, with papillae extending outward for 0.1–0.12 mm (Fig. 3). Both cell types discharge their contents when the proboscis is in use, their secretions showing a similar appearance to those described for the foregut.

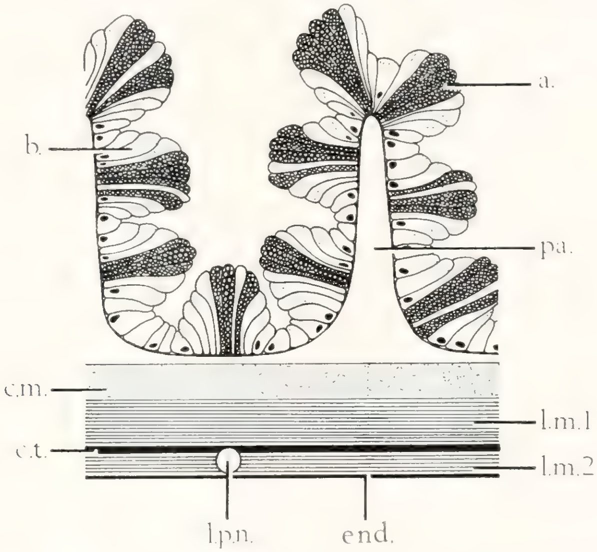


FIGURE 3. *Paranemertes peregrina.* Diagrammatic representation of the structure of the anterior proboscis when in the protruded position; a., acidophilic gland cell; b., basophilic columnar cell; c.m., circular muscle layer; c.t., connective tissue; end., endothelium; l.m.1, inner longitudinal muscle layer; l.m.2, outer longitudinal muscle layer; l.p.n., longitudinal proboscis nerve; pa., parenchyma.

Beneath the epithelium parenchymatous tissue forms a layer that extends into the papillae to form a semi-rigid core. The parenchyma overlies three muscle zones consisting of a single layer of circular fibers and two outer layers of longitudinal fibers. The longitudinal muscles are separated by a narrow zone of connective tissue. Enclosing the anterior proboscis a thin endothelium lies next to the outer longitudinal muscle layer.

The proboscis of *Paranemertes* is furnished with fourteen longitudinal nerves that extend the full length of the anterior region in the outermost muscle layer and its adjoining connective tissue.

At its posterior end the anterior proboscis narrows into the central muscular bulb, which is divided into two parts. The anterior region contains the stylet apparatus. The single central stylet, 80 μ long, is carried on a cylindrical waisted base of about 100 μ length and 50 μ maximum diameter. On either side of, and slightly anterior to, the central stylet are the paired accessory stylet pouches, each containing from two to eight accessory stylets of similar size to the central one. Both central and accessory stylets show the braided or fluted effect described by Coe (1905) for the more southerly Californian variety of *Paranemertes peregrina*.

The posterior half of the stylet bulb consists entirely of muscle fibers arranged around a sac-like central lumen, and it is probably through the contractions of these muscles that the proboscis secretions are forced into the body of the prey via the stylet wounds. The bulb lumen connects the anterior and posterior proboscis chambers by narrow canals (Fig. 2).

The third, posterior, region of the proboscis consists of a thin endothelium enclosing a single muscle layer of longitudinal and oblique fibers. Bordering the musculature a parenchymatous layer lies below the inner epithelium consisting of interstitial and gland cells that are not arranged into papillae. Glands, 15–20 μ long and 5–8 μ wide, are filled with irregular-shaped proteinaceous acidophilic granules of maximum dimension about 1.5 μ. There are no basophilic components in this proboscis region, and the interstitial cells possess no particular staining affinities. The proboscis lumen is often partly filled with a coarsely granular matrix that is secreted by the gland cells, and soon after proboscis eversion has taken place the lumenar contents show an increase in density at the same time as the glands can be seen discharging their contents.

The functions of the proboscis

It seems evident that the two major proboscis regions possess distinctive roles in the function of the organ. When the proboscis is protruded the central stylet is terminal (Roe, 1967), and any secretions poured into the wound inflicted by the stylet, forced from the proboscis by the contractions of the muscular bulb, can only arise from the posterior gland cells (Fig. 2b). Immobilization of nereid prey does not occur in the absence of stylet penetration, and it must therefore be presumed that the paralytic toxins are produced and secreted only by the posterior proboscis epithelium. Any comparable substances discharged from the anterior proboscis papillae would be both distant to the stylet wound, and subject to dilution from the surrounding sea water. However, these theoretical considerations are at variance with the findings of W. R. Kem, University of Illinois, unpublished results, who reports that the anabaseine toxin comprises some 7% of the wet tissue weight of the anterior proboscis, as well as being secreted by the general epidermis. This author fails to comment on the distribution of the toxin in the posterior proboscis and, in the light of this conflicting evidence, it seems inadvisable to draw further conclusions until additional investigations have been conducted.

The papilliary secretions, as noted earlier, closely resemble in appearance those of the foregut. The structure of the anterior proboscis suggests that the papillae assist in the organ gripping its catch, and it is likely that some of the secretions at least are viscous and play a supplementary role. Both non-specific esterase and acid phosphatase activity have been recorded in the acidophilic gland cells, with the former appearing additionally in the papilliary secretions. Since the basophilic secretions are distinctly "stringy" in appearance, it may be suggested that it is these that comprise the viscous component of the papilliary products, and that the gland cell esterases possess other roles. The precise function of the enzymes has not been established, but they may be involved in either initiating the disruption of the polychaete epidermis or in enhancing the viscous properties of the basophilic secretions. There is no evidence to suggest that *Paranemertes* possesses an extracorporeal digestive phase, as reported for other hoplonemertean species by Jennings and Gibson (1969).

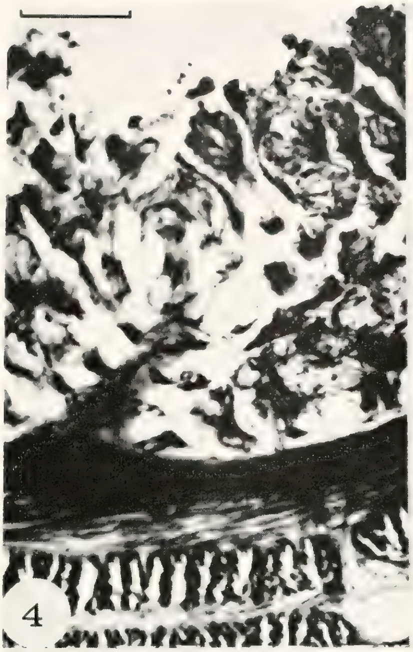
The site and sequence of digestion

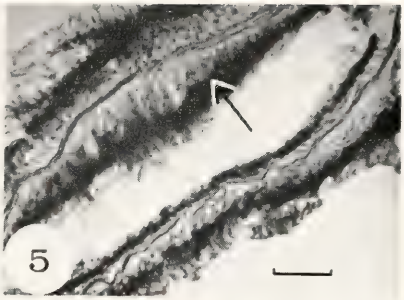
The food and feeding mechanism of *Paranemertes* have been fully reported by Roe (1967). Nereid polychaetes are caught by the proboscis, immobilized by its secretions, and ingested whole by means of a sucking action. At this time the foregut actively discharges its secretions, which have an acidic pH value of 5.5–6.0, as determined from *Platynereis* specimens examined during ingestion after having been previously stained with indicator dyes. No carbonic anhydrase activity was ever observed in any part of the gut, so the acid secreting mechanisms must involve other enzymes, presumably associated with the proteinaceous acidophilic glands. The role of the foregut basophilic secretions is not clearly understood, but two possibilities may be suggested. First, they may possess a lubricative function to facilitate ingestion or, second, they may activate the acidophilic secretions within the gut lumen.

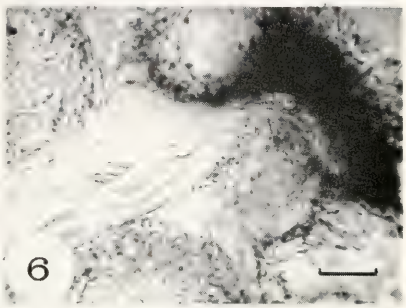
Food material thus enters the intestine in an acidic medium, the acid secretions serving both to kill the prey and provide the appropriate pH level for subsequent extracellular proteolysis. As the food enters the intestine the gastrodermal gland cells discharge their contents. At the same time there is an increase in the number of the acidophilic inclusions within the columnar cells and they are discharged into the gut lumen between the cilia. As noted earlier, these inclusions contain cathepsin-C like endopeptidases (Fig. 5) and the amount of intralumenar activity of these enzymes increases as more of the inclusions are discharged.

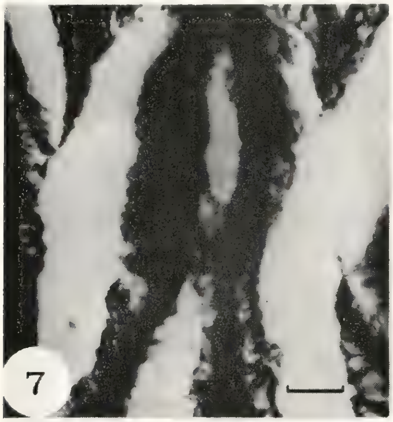
Within two hours of a meal the food is sufficiently broken up to allow the phagocytosis of food particles by the columnar cells, food vacuoles formed at this time showing strong endopeptidase activity. There is no evidence to suggest that endopeptidases are secreted cytoplasmically, so that the intracellular activity observed presumably originated from the gut lumen, being taken into the cells during the phagocytotic processes. During this digestive phase strong acid phosphatase activity can be demonstrated in and around the food vacuoles (Fig. 6), where it is presumed to be concerned in some way with the intravacuolar maintenance of the acidic pH necessary for the efficient functioning of the proteases.

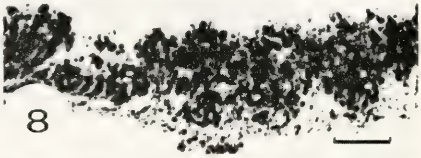
As time progresses the number of food vacuoles increases and six hours after a meal the columnar cells are packed with them. The early phase of intracellular

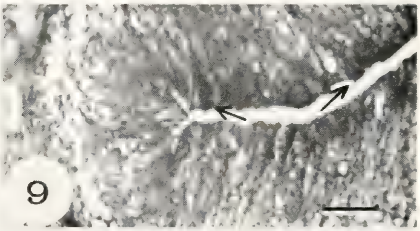


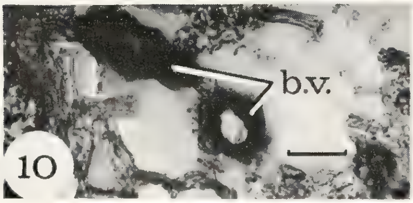












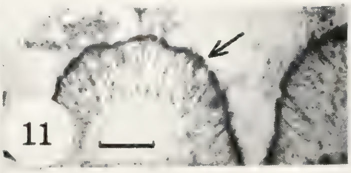


FIGURE 4. *Paranemertes peregrina.* Transverse section through the anterior proboscis in retracted position, showing how the cellular arrangement of the papillae is obscured; compare with Figure 3; Mallory; scale: 20 μ.

digestion lasts for up to thirty-six hours or more, by which time the residues of the meal have been evacuated from the gut lumen. Following this there is a decline in the amount of demonstrable acid phosphatase and endopeptidase activity, these enzymes becoming replaced by exopeptidases and alkaline phosphatase at the same sites. The activity of these enzymes persists within the columnar cells until digestion is completed, when the exopeptidases disappear and cannot be further demonstrated until the appropriate stage of a subsequent meal. This is the usual pattern of exopeptidase activity as reported for other nemertean species. Alkaline phosphatase activity, which is intense during peak exopeptidase visualization (Fig. 7), is at all other times present only as a faint zone of activity at the distal margins of the columnar cells. The enzymes responsible for the second, alkaline stage of intracellular digestion can be demonstrated within food vacuoles and their surrounding cytoplasm for as long as ninety-six hours after a meal.

Carbohydrases and lipases were not demonstrated at any stage in digestion, but the failure to visualize their activity could be explained by the relatively low amounts present. Lipases, for example, have only been successfully demonstrated in specimens fed on a high fat diet (Jennings, 1962a), and carbohydrases have been reported only by inference (Jennings and Gibson, 1969). There is no reason to suppose that *Paranemertes* in any way differs with respect to its carbohydrate- and fat-digesting enzymes.

The food reserves

Fat forms the principal food reserve in *Paranemertes*, being stored mainly in the gastrodermal columnar cells as globules of 2 μ or less diameter (Fig. 8). Occasional deposits up to 7 μ across can be found, but these are irregularly distributed. Fat is deposited more or less uniformly throughout the cell cytoplasm, but tends to be absent from a distal zone 5–8 μ deep.

No fat deposits were found in the parenchyma, epidermis, foregut, blood system or body musculature, but occasional globules of 1.5–2 μ diameter were observed

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FIGURE 5. *Paranemertes peregrina.* Section through the gastrodermis of a starved specimen showing columnar cells with the acidophilic spheres which are the sites of endopeptidase activity (arrowed); Hess and Pearse method; scale: 60 μ.

FIGURE 6. *Paranemertes peregrina.* Part of the gastrodermis six hours after a meal showing the distribution of acid phosphatase activity (black) in and around food vacuoles; Burstone's azo dye method; scale: 30 μ.

FIGURE 7. *Paranemertes peregrina.* Oblique section through the gastrodermis during the later stages of intracellular digestion to show the intense alkaline phosphatase activity (black) distributed throughout the columnar cell cytoplasm and in food vacuoles; Gomori's calcium salt method; scale: 60 μ.

FIGURE 8. *Paranemertes peregrina.* Longitudinal section through part of the gastrodermis to show the distribution of fat globules; Flemming; scale: 12 μ.

FIGURE 9. *Paranemertes peregrina.* Transverse section of a part of the gastrodermis showing the restriction of glycogen storage to the distal regions of the columnar cells (arrowed); Bauer; scale: 20 μ.

FIGURE 10. *Paranemertes peregrina.* Section through two blood vessels (b.v.) to show the intense "leucine aminopeptidase" activity consistently present in their lining walls; Burstone and Folk method; scale: 10 μ.

FIGURE 11. *Paranemertes peregrina.* Longitudinal section through the body wall showing the narrow distal zone of non-specific esterase activity (arrowed); Gomori's -naphthyl acetate method; scale: 20 μ.

in the anterior proboscis, where they were restricted to the connective tissue separating the two layers of longitudinal muscle fibers.

At the time of collection mature specimens were not available, so no observations on fat deposition within the ova and ovarian endothelia can be made. These sites usually contain large amounts of fat in other species.

Small amounts of glycogen, occurring as tiny scattered granules, are stored in the distal regions of the gastrodermal columnar cells (Fig. 9), the body wall muscles (particularly the longitudinal layers), and in the parenchyma adjoining the gastrodermis.

Other sites of enzymic activity

Strong exopeptidase activity is consistently present in association with the blood vascular system (Fig. 10), a regular site for this enzyme previously reported in other species by Gibson and Jennings (1967).

Variable amounts of non-specific esterase activity were found in several other sites in the body, demonstrable by both the indoxyl acetate and α-naphthyl acetate techniques. At each site the intensity of activity is independent of the nutritive state.

Weak esterase activity was observed in the rhynchocoel endothelium, longitudinal body musculature, and endothelium and plasma of parts of the blood vascular system. Irregular, but stronger amounts were visualized in the connective tissue separating the two longitudinal muscle layers of the anterior proboscis, and in the outer sheath of parts of the main lateral nerve rords. The strongest esterase activity appeared in the tracts of the cerebral glands, and as a 2.5–4 μ thick distal border to the epidermis (Fig. 11).

The only other enzymic activity demonstrated was acid phosphatase, parallelling the non-specific esterase distribution in the epidermis.

Discussion

The digestive physiology of *Paranemertes peregrina* closely resembles that described for other nemertean species by Jennings and Gibson (1969). These authors showed that amongst hoplonemerteans interspecific differences occur principally with respect to the acid-secreting mechanisms of the foregut, specifically in the presence or absence of demonstrable carbonic anhydrase activity in the acidophilic gland cells. The occurrence of this enzyme appeared to be restricted to those species in which direct ingestion of the food was not preceded by an extracorporeal digestive phase, this being true for palaeo- and heteronemerteans also. *Paranemertes* thus differs from most other nemerteans in lacking both extracorporeal digestion and demonstrable carbonic anhydrase activity, although its foregut secretions are clearly acidic in nature. A similar situation is found in the bdellonemertean *Malacobdella grossa,* but physiological and morphological differences in this species can be related entirely to its atypical commensal habits (Gibson and Jennings, 1969).

Paranemertes is less closely related to *Amphiporus* and *Tetrastemma,* which lack carbonic anhydrase, than is *Prostoma,* which possesses the enzyme. This suggests that amongst these species variations in foregut physiology cannot simply be related either to systematic position or feeding mechanism, and it can be concluded only that this aspect of hoplonemertean digestive physiology is subject to interspecific alteration whose controlling factors are not yet understood.

The absence of a distinct esophagus and pyloric tube from *Paranemertes* is much more likely to be related to the manner of feeding. A similar absence of foregut differentiation is found in anoplan species where the food is ingested directly in a manner like that described for *Paranemertes* by Roe (1967, 1970). In contrast, hoplonemertean species such as *Amphiporus*, in which the foregut is partially protruded for feeding, possess not only a distinct oesophagus and an extended pyloric tube, but also show much more folding of the foregut epithelium than is found in species feeding in the more conventional nemertean manner.

As in other carnivorous nemerteans, the emphasis in *Paranemertes* is placed upon the production of proteolytic enzymes by the gut, those acting extracellularly being secreted by the gastrodermal columnar cells in the usual hoplonemertean fashion. The gland cells also discharge their products into the intestinal lumen, but the nature of their secretions remains undetermined. It is possible that they represent additional proteolytic enzymes not demonstrable by the techniques employed in the present work.

The persistent zone of alkaline phosphatase activity in the gastrodermal border may be involved in the phagocytosis and absorption of food material from the gut lumen. These enzymes have been linked with the phosphorylative transfer of extracellular substances by Danielli (1952) and Erasmus (1957), and Halton (1967) has reported that in polyopisthocotylean Monogenea alkaline phosphatases are found distally in the gastrodermis during the absorption of food materials. Similar roles have been attributed to these enzymes in other nemerteans (Jennings, 1962a; Jennings and Gibson, 1969; Gibson and Jennings, 1969), in archiannelids (Jennings and Gelder, 1969), and in rodents (Hugon and Borgers, 1968). The enzymes may further be concerned with the uptake of lipids, since Noma (1964) and Raghavan and Ganguly (1967) have demonstrated that shortly after a meal and during the active absorption of materials there is an increase in the phospholipid content of the intestinal mucosa.

Of the enzymes found at sites other than in the gut, exopeptidases consistently present in the blood vascular system are believed to be involved in the circulation of amino acids and simple peptides, as discussed by Gibson and Jennings (1967).

The role of the epidermal enzymes has not been established, but they may be concerned in one or both of two distinct mechanisms. W. R. Kem (unpublished) recorded that 70% of the species' toxin is localized within the epidermal tissues, and the enzymes may thus play a part in the secretion of this substance as a defensive mechanism against predation. Distasteful epidermal secretions at least are apparently produced by other species, since Gibson (1968) recorded that the extremely voracious littoral fish *Parenophrys bubalis* consistently refused to feed on lineids, even when starved and readily prepared to accept a wide variety of other natural and artificial foods.

A somewhat more plausible explanation of the role of these enzymes concerns the uptake of simple nutrient materials from the environment. Fisher and Cramer (1967) showed that glucose and amino acids were absorbed across the epidermis of *Lineus ruber*, and concluded that the epidermal microvilli were involved in this process. Jennings and Gibson (1969), in reporting the occurrence of epidermal enzymic activity in a number of nemertean species, suggested that these enzymes may be concerned with the extracorporeal digestion of simple proteins or polypeptides, which could then be absorbed across the microvilli. This postulated link between epidermal enzymes, microvilli and absorption is further discussed by Jennings (1969), who comments on the similarity between gastrodermal and epidermal microvilli, although noting that the former are not apparently concerned in the normal phagocytotic processes of the gut. The epidermal absorption of nutrient materials may thus be a general feature of nemerteans, in which case the blood system exopeptidases may well be additionally involved in this mechanism.

The roles of other non-digestive enzymes are far from being understood. In the anterior proboscis connective tissue an association between the non-specific esterases and fat reserves may tentatively be drawn, as certain esterases are involved in the hydrolysis of triglycerides (Reid and Dunnill, 1969), and are believed to be partly responsible for intracellular lipid metabolism in bdellonemerteans (Gibson and Jennings, 1969). The absence of demonstrable lipases from these sites is, however, at variance with this suggestion.

Whether a similar role can be envisaged for esterases in other tissues is in doubt, particularly since fat deposits are otherwise restricted to the gastrodermis. Certainly in the cerebral glands, where strong esterase activity is persistently recorded, the enzymes must function in other metabolic processes. It is not known for certain whether these glands are involved with chemotactic (Reisinger, 1926), endocrine (Scharrer, 1941) or other functions.

The author wishes to extend his sincere appreciation to Dr. R. L. Fernald for providing facilities at the Friday Harbor Laboratories.

SUMMARY

1. Digestion in the hoplonemertean *Paranemertes peregrina* is achieved by a combination of extra- and intracellular processes. The extracellular phase, effected in an acidic medium, involves endopeptidases secreted by the gastrodermal columnar cells, and other, as yet unidentified, substances discharged from the intestinal gland cells. The semi-digested food is then phagocytosed and digestion completed intracellularly by peptidases, carbohydrases and lipases acting in harmony. Intracellular digestion is initially acid and then alkaline, with acid and alkaline phosphatases associated with the appropriate phases.

2. Nereids used as food are caught by the proboscis, and immobilized by secretions produced by the posterior proboscis gland cells. These secretions are pumped into the body of the prey via wounds caused by the central stylet. The nature of these secretions has not definitely been established, but they may contain the toxin anabaseine.

3. The anterior proboscis secretions are concerned with aiding the grip of the proboscis papillae and possibly with initiating the denaturation of the prey epidermis.

4. Acid secretions are produced by the foregut via a mechanism that does not involve carbonic anhydrase.

5. Other sites of enzymic activity have been reported, and where possible suggestions made as to their probable roles.

6. Fat forms the principal food reserve, with major deposits being stored in the gastrodermal columnar cells, but some glycogen is stored in a variety of body tissues.

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ENVIRONMENTAL TEMPERATURE AND THYROID ACTIVITY IN THE LIZARD, *SCELOPORUS OCCIDENTALIS*

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A relation between environmental temperature and the secretory activity of the thyroid gland has been reported for a number of species of lizards on the basis of both field and laboratory studies (see reviews, Lynn, 1960, 1970). It has been suggested that the influence of temperature change upon the thyroid is an indirect one, mediated by the hypophysis (Eakin, Stebbins and Wilhoff, 1959). Increase in temperature is assumed to result in a rise in the release of TSH from the hypophysis and decrease in temperature in a lowering of TSH release. The present expriments were designed to further investigate the relationship between temperature and the hypophyseal-thyroidal axis by a study of radioiodine incorporation by the thyroid in intact, hypophysectomized and sham-hypophysectomized animals maintained at various temperatures ranging from 15° to 38° C and by examining the effects of TSH administration to hypophysectomized animals at the highest temperature, 38° C.

Materials and Methods

A total of 200 specimens of *Sceloporus occidentalis* (Baird and Girard) were purchased from a supply company in California. Before being used for experimentation, the animals were kept for at least one week at room temperature (26 to 33° C) with an 8-hour daily period of illumination. They were fed with larval and adult specimens of *Tenebrio* and water was available to them at all times.

At the end of this period, fully adult lizards of fairly uniform weight were selected and these were divided into groups placed in constant temperature chambers kept at (a) 21 ± 2° C and 30 ± 1° C (experiments performed in October, 1968) and (b) 15 ± 1° C, 30 ± 1° C and 38 ± 1° C (experiments performed in September, 1969). This choice of temperatures was guided by the fact that they cover the range from minimum to maximum voluntary temperatures reported for the species (Brattstrom, 1965; Cunningham, 1966; McGinnis, 1966). After one week in the constant temperature chambers, each group was subdivided into three sets of animals as follows: (A) intact, untreated animals; (B) animals with the pars distalis of the hypophysis removed; (C) sham operated animals. The number of specimens in each group varied and will be indicated in the results (Table I).

At the end of the second week at the designated temperature (*i.e.*, one week after operation), each animal was given an intraperitoneal injection of 5 μc of carrier-free I^{131}. The level of radioactivity in the thyroid region was then measured at 24-hour intervals for a period of seven days.

TABLE I

The percentage uptake of I^{131} *by the thyroid gland of the western fence lizard,* Sceloporus occidentalis

Mean ± S.E.

Days after I^{131} inj.	Untreated controls				Sham-operated controls				Pars distalis removed			
	38 ± 1°	30 ± 1°	21 ± 2°	15 ± 1°	38 ± 1°	30 ± 1°	21 ± 2°	15 ± 1°	38 ± 1°	30 ± 1°	21 ± 2°	15 ± 1°
	[4]	[9]	[4]	[6]	[5]	[8]	[4]	[4]	[6]	[9]	[8]	[6]
1	16.0 ± 0.5	19.9 ± 3.5	19.1 ± 3.9	2.0 ± 0.4	19.2 ± 1.0	21.2 ± 3.9	25.3 ± 2.5	2.6 ± 0.4	2.4 ± 0.7	8.5 ± 2.1	8.8 ± 1.9	2.8 ± 1.5
2	22.1 ± 7.3	33.1 ± 5.8	35.0 ± 5.5	3.9 ± 0.8	30.8 ± 4.9	33.3 ± 5.4	41.5 ± 3.7	6.0 ± 0.6	3.7 ± 1.4	13.4 ± 3.0	14.2 ± 3.0	7.1 ± 3.5
3	27.1 ± 5.6	41.8 ± 5.4	43.4 ± 8.5	6.2 ± 1.4	34.7 ± 6.7	41.4 ± 5.8	52.8 ± 4.0	8.8 ± 0.5	3.5 ± 1.7	16.7 ± 4.0	19.1 ± 4.4	10.1 ± 4.9
4	30.8 ± 10.2	47.6 ± 5.8	50.5 ± 7.8	7.2 ± 2.0	37.8 ± 9.1	46.8 ± 5.6	59.1 ± 5.3	10.6 ± 0.4	4.1 ± 2.3	19.1 ± 4.4	22.2 ± 4.8	13.1 ± 6.1
5	32.2 ± 12.0	50.7 ± 5.7	55.7 ± 8.3	9.7 ± 2.2	46.6 ± 3.1	49.4 ± 5.5	64.1 ± 5.9	12.7 ± 0.2	4.5 ± 2.4	20.8 ± 4.6	26.7 ± 5.9	15.4 ± 7.1
6	34.2 ± 13.4	52.6 ± 5.6	57.8 ± 7.6	10.7 ± 2.6††	51.4 ± 0.6	51.6 ± 5.8	68.6 ± 5.4	15.1 ± 0.6	5.1 ± 2.7*†	22.0 ± 4.8**	29.9 ± 7.7**	17.4 ± 8.3***
7	35.6 ± 14.9	54.2 ± 5.8	61.9 ± 8.3	12.6 ± 2.9	52.6 ± 2.2	51.7 ± 5.5	69.8 ± 4.1	16.1 ± 1.3	5.4 ± 3.0	22.3 ± 4.8	31.3 ± 7.9	20.4 ± 9.1

Figures in brackets refer to number of animals.

* Animals with pars distalis removed versus sham-operated controls $P < 0.001$.

** Animals with pars distalis removed versus sham-operated controls $P < 0.01$.

*** Animals with pars distalis removed versus sham-operated controls P = not significant.

† Animals with pars distalis removed: 38 ± 1° versus 30 ± 1° $P < 0.02$.

†† Untreated control: 15 ± 1° versus 30 ± 1° $P < 0.001$.

Measurements of radioactivity were made with a scintillation counter consisting of a $1\frac{3}{4} \times 2$-inch NaI crystal, an RCA 6342A photomultiplier tube with a conventional amplifier, and a binary scaler. The crystal and photomultiplier were mounted in a lead cylinder 5.2 mm thick with a collimating slit measuring 4.0×120 mm. This was large enough to cover the area of the thyroid when the ventrum of the animal was properly placed over the slit. The animals remained in the constant temperature chambers throughout the week-long counting period during which they were not fed because it had previously been found that these animals show variations in thyroid activity levels during digestion.

To make a statistical comparison of the percentage I^{131} uptake by the thyroid in the various groups, we have used the mean value of the counts ± the standard error of the mean for each group on the sixth day after radioiodine administration ("t" test). This seems justifiable because we have found in previous experiments that the maximum uptake is usually reached by the fifth day and then remains at approximately the same level for about one week.

The results of these experiments indicated the desirability of a further study to test the effects of TSH administration to hypophysectomized animals. Specimens remaining in stock were used for this experiment. They were kept at 38 ± 1° C for one week, removal of the pars distalis was then carried out, and beginning 24 hours after the operation, all animals were given daily injections of 0.025 USP units of TSH per gram body weight in 0.015 ml of 0.7% saline, or just the saline without TSH, for 7 days. Three hours after the last TSH injection, each animal was given 5 μc of carrier-free I^{131} and the radioactivity in the thyroid region was determined at 24-hour intervals for one week as described above. It is difficult to say whether the TSH dosage used is a "physiological" one for lizards since nothing is known of the secretion rate of the hormone in reptiles. It was chosen because our preliminary studies indicated that it resulted in I^{131} uptakes in hypophysectomized animals that were comparable to those found in normal, unoperated lizards.

Surgical removals of the pars distalis and sham-operation were performed under anesthesia with sodium pentabarbital (Nembutal, Abbott). The TSH was purchased from Nutritional Biochemicals Corporation and was reconstituted with 0.7% saline to a concentration of 10 USP units per ml.

RESULTS

General observations

Lizards kept at 15° C became sluggish within a short time and remained inactive throughout the experiment. Those at 21° C were quiescent but, unlike those at 15° C, would move about for short periods when disturbed. At 30° and 38° C the lizards were highly active and very excitable, especially at the latter temperature. Only five specimens of the 73 used in this experiment died during the course of the work. One of these was a sham-operated animal kept at 15° C. The other four were lizards kept at 38° C, two of the four untreated controls and two of the five sham-operated animals.

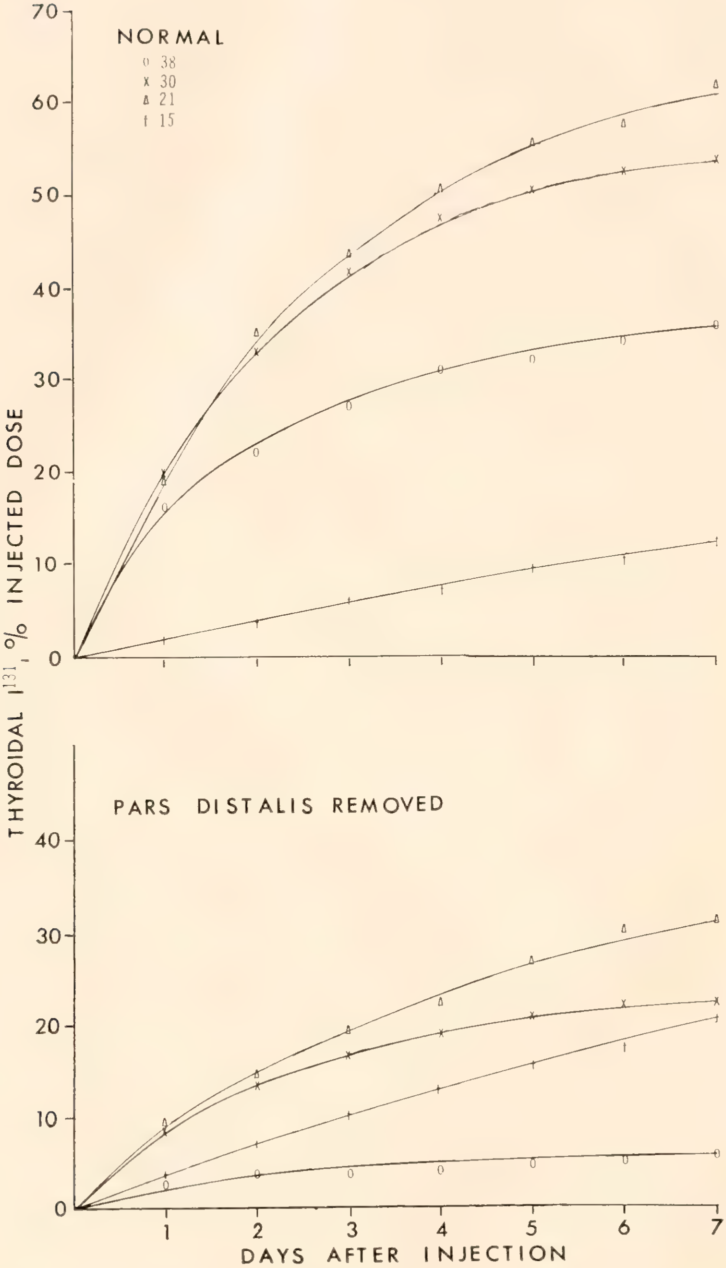

FIGURE 1. Radioiodine uptake by the thyroid, in per cent of injected dose, at various temperatures in unoperated *Sceloporus* and in those with the pars distalis of the hypophysis removed.

I^{131} accumulation by the thyroid

The percentage of I^{131} incorporated by the thyroid gland on successive days after injection is given in Table I for all groups except those given TSH treatment and these data are shown graphically in Figure 1. The data recorded for 30° C represent combined figures for an experiment carried out in two different years (Oct. 1968 and Sept. 1969). Statistical analysis showed no significant difference for these two groups of animals and it has seemed justifiable to combine the figures in the interest of brevity. It will be noted that intact, untreated controls kept at any of the high temperatures (21°, 30° or 38° C) showed a gradual rise in radioactivity in the thyroid region during the first 5 days after administration of the isotope and then a levelling off during the sixth and seventh days. However it is noteworthy that the maximum levels reached did not differ significantly at these three temperatures. Although the maximum figure for 38° C appears to be lower than those for 21° C and 30° C, statistical analysis shows that this difference is not significant ($P > 0.8$). At the lowest temperature (15° C), the untreated

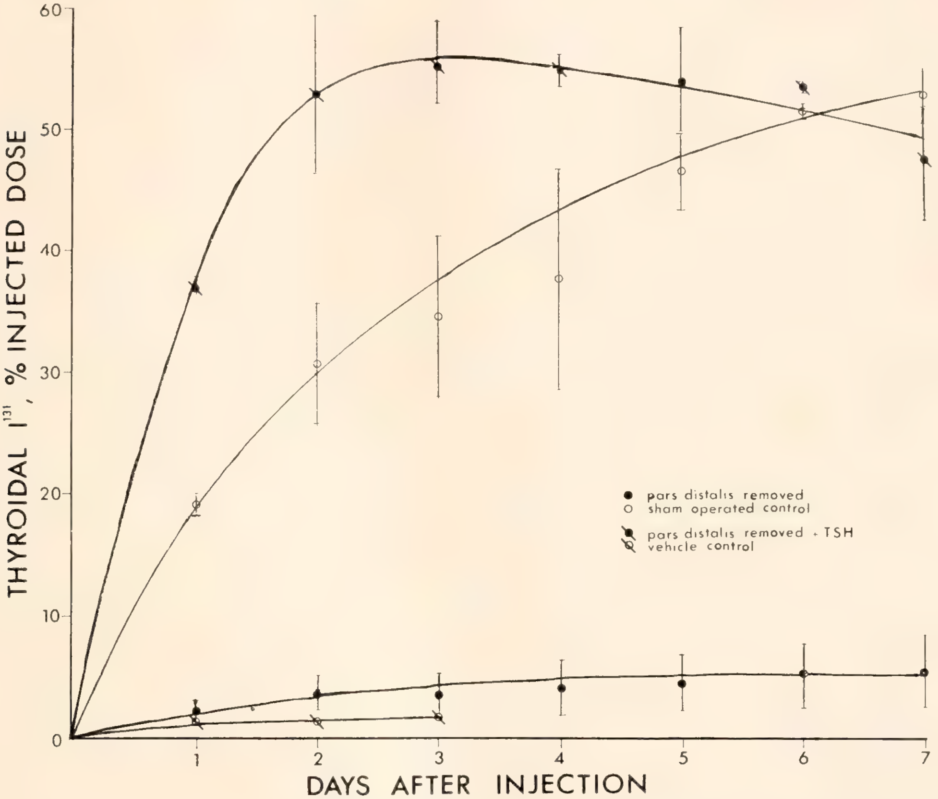

FIGURE 2. The effect of TSH administration upon radioiodine uptake by the thyroid, in per cent of injected dose, in *Sceloporus* with pars distalis removed and in sham-operated animals.

controls showed a much lower rate of accumulation and no tendency to level off during the 7-day counting period. This was also true for sham-operated animals and operated animals maintained at this temperature. Comparison of the figures for untreated controls with those for sham-operated animals shows that the sham-operation had no significant effect upon I^{131} uptake by the thyroid. Surgical removal of the pars distalis of the hypophysis resulted in a significant decrease in thyroidal accumulation by the thyroid at all of the high tempertures (21°, 30° and 38° C) but hypophysectomized animals kept at 15° C showed no significant difference in radioiodine uptake as compared with intact or sham-operated animals at that temperature.

The percentage uptake of I^{131} by the thyroids of hypophysectomized lizards injected with TSH is shown graphically in Figure 2. It may be seen that TSH administration resulted in a great increase in radioiodine accumulation especially during the first two days after injection of the isotope, which reached a maximum by the third or fourth day and then showed a slight decline.

DISCUSSION

The present results indicate a general pattern of I^{131} accumulation in the thyroid of *Sceloporus occidentalis* which is similar to that found for other squamate reptiles such as *Gekko* (Chiu *et al.*, 1967) and *Thamnophis* (Chiu and Lynn, unpublished) and for the turtles *Pseudemys* and *Terrapene* (Shellabarger *et al.*, 1956). However, it should be noted that other experiments on *Sceloporus occidentalis* and *Anolis carolinensis* (Kobayashi and Gorbman, 1959) and on *Xantusia henshawi* (Buckingham, 1970) have shown a more rapid rise in thyroidal radioiodine to reach a maximum value by the second day after I^{131} injection with a retention at this value for at least six more days, and still another study on *Anolis carolinensis* (Lynn *et al.*, 1965) indicates that, after reaching maximum levels at about two days, the levels fall rapidly over the next four days, especially in lizards kept at high temperature (35° C).

Comparison of data on radioiodine uptake by the thyroid in intact, untreated animals at the various temperatures used in the present study shows, rather surprisingly, that over the range of 21° C to 38° C, there is no evidence of any significant temperature-related change in thyroid activity. Only at 15° C is the thyroid functioning significantly inhibited. This would seem to indicate that there is a certain critical temperature, somewhere between 15° C and 21° C below which the thyroid gland is relatively inactive but that above this critical temperature the gland reaches a level of activity that is not modified by further temperature increase, at least over the 21° C to 38° C range. It appears that this finding by itself would oppose the theory of a direct effect of temperature change upon the thyroid in the sense of a regular increase in physiological activity with every temperature rise.

Moreover, it is well known that the functioning of the thyroid is under the control of a hypophyseal thyrotropic hormone. This has been demonstrated for the species used in these experiments (Gorbman, 1946; Eakin *et al.*, 1959), as well as for other lizards such as *Anolis* (Nussbaum, 1963; Lynn *et al.*, 1965) and *Xantusia* (Buckingham, 1970). In the present study, as shown by the graphs for the animals with the pars distalis of the hypophysis excised, absence of the hypophyseal hormones results in a sharp decrease in thyroid activity in all animals except those kept at 15° C. This supports all previous studies on the importance of TSH in normal thyroid functioning in lizards, and also indicates that at 15° C the thyroids of intact, untreated animals are functioning at no higher level than are those of animals that completely lack TSH.

Wilhoft (1958) and Eakin *et al.* (1959) found that fence lizards kept at 35° C are much more active than those kept at room temperature and advanced the hypothesis that this is related to increased thyroid activity at higher temperature which is, in turn, due to a heightened release of TSH from the hypophysis. In view of the present results, it seems more likely that the role of temperature in thyroid activation is simply a permissive one. Below a certain temperature level there is no response to temperature change; above that level the thyroid is active regardless of the precise temperature, at least over the 21° to 38° C range. This is supported also by work done in this laboratory on *Phrynosoma* (Leichner, unpublished). This would mean either that TSH release from the hypophysis is subject to an "all or none" relation to temperature or, alternatively, that the responsiveness of the thyroid to TSH stimulation is so related.

Despite the small number of animals available, it seems clear that the dosage of TSH used (0.025 USP units per gram body weight daily for seven days) was sufficient to increase the uptake of radioiodine by the thyroids of hypophysectomized specimens to approximately the normal level for intact animals maintained at the same temperature (38° C). However, it must be emphasized that, although the level of uptake reaches similar values in these groups, the precise pattern of radioiodine accumulation in the hypophysectomized animals given exogeneous TSH at a single dosage level is quite different from the normal pattern. In the latter the accumulation is gradual over the seven-day counting period, whereas in the former the highest level is reached by the third day and then declines. This raises the interesting possibility that there is normally a continuous and constant rate of release of TSH from the hypophysis in many squamate and turtle species which accounts for the pattern of thyroidal accumulation of I^{131} in *Gekko, Thamnophis, Terrapene* and *Pseudemys,* and *Sceloporus.* When a cyclic fluctuation in endogeneous TSH production and release occurs, a different pattern of thyroidal I^{131} accumulation comparable to those reported for *Anolis* (Lynn *et al.,* 1965) and *Xantusia* (Buckingham, 1970) would result. In the present study, the pattern found for hypophysectomized *Sceloporus* receiving TSH injections would also be explicable on this basis.

Summary

The relationship between temperature and the hypophyseal-thyroidal axis in *Sceloporus occidentalis* has been investigated by a study of radioiodine incorporation by the thyroid in intact animals, and animals with the pars distalis removed, kept at temperatures of 15°, 21°, 30° and 38° C. Over the range of 21 to 38° C there was no evidence of any significant temperature-related change in the normal and experimental animals, although the thyroid activity was reduced after the operation. At 15° C the thyroid functioning was significantly inhibited and there was no difference between the normal and experimental animals. It is suggested that the role of temperature in thyroid activation is a permissive one. Data for

I^{131} incorporation in hypophysectomized animals receiving TSH therapy offers a possible explanation for the different patterns of thyroidal I^{131} accumulation observed in different forms of lizards.

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EMERSION OF THE AMPHIBIOUS CHILEAN CLINGFISH, *SICYASES SANGUINEUS*

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Amphibious fishes ". . . spend periods of time out of water, on or above the ground surface, as normal parts of their life histories" (Gordon, Böetius, Evans, McCarthy, and Oglesby, 1969, page 141). Many inhabit marginal zones like the marine intertidal zone, which fluctuates between aquatic and terrestrial conditions. Some gobies and blennies briefly expose themselves as they scurry from one tidal pool to another; others may remain out of water for many hours while the tide is out, keeping moist in damp seaweed or under rocks (Gordon, 1966). William H. Eger (in preparation) found several small species of clingfishes (Gobiesocidae) of the Gulf of California in moist areas under dry rocks or in clumps of snails far from the water's edge.

Sicyases sanguineus of coastal Chile and southern Perú is an exceptionally large clingfish which can live both below the cool and well-aerated surf and above water on exposed rocks (Buen, de, 1960). This Chilean clingfish attaches by means of a large disc formed of the fused and highly modified pelvic fins, bearing small and flattened dermal papillae (Briggs, 1955). The complementary forces of suction by the disc and adhesion by the papillae secure this clingfish to the rock, so that it resembles a large chiton or limpet blending with the dark substrate. Although young fish are relatively active as they occasionally scamper in and out of the water, adults are quiescent as they cluster on exposed rocks often well above water level (Vargas and Concha, 1957ab).

To survive above the surf, clingfish must (1) either tolerate large fluctuations of the environment or remain in the splash zone and (2) breathe air. If they leave the splash zone, they risk variable oxygen concentration, temperature, and salinity in isolated tidal pools, and desiccation and overheating on drying rocks (*cf.* Carter, 1931). If emerged fish were continuously wetted and cooled in the splash zone, however, they could survive with minimum adjustments, other than those necessary for breathing air. With this in mind, we set out: (1) to determine the optimal circumstances of the Chilean clingfish's life above water; (2) then, to find out why these circumstances generally prevail and if they can be largely compromised; which (3) led to experimental studies of the mechanism of the fish's aerial respiration. Our investigations supplemented more intensive physiological studies by Gordon, Fischer, and Tarifeño (in preparation) of the survival of the fish out of water.

MATERIALS AND METHODS

Field observations

We observed the behavior and distribution of fish at three localities along the coast of central Chile, north of Valparaíso in a subtropical region of light to moderate rainfall. At each locality fish were watched through binoculars as they clung to the vertical surfaces of three large rocks on progressively higher ground: rock 1 with base awash at low tide, to rock 3 with base awash only at high tide. These surfaces faced mostly southwest, so that they were shaded in the morning and sunlit in the afternoon. They either paralleled the surge or formed a lee, protecting the fish from the full force of the surf. Locality I was in a relatively urban area just north of the Estación de Biología Marina at Montemar, while localities II and III were in sparsely populated rural areas about 60 km north of I.

The observed surfaces of rocks 1 measured about four square meters at localities I and III, and about six square meters at locality II; rocks 2 and 3 together measured barely three square meters at all localities. The surface of rock 1 was subjectively divided into two habitats: a lower half generally wet at low tide and an upper half generally dry at low tide. A fringe of brown algae about one meter above tidal low underlaid the surfaces of rocks 1 and 2. With approaching high tide, larger swells broke over the rocks to form characteristic rivulets down the sheltered surfaces. Clingfish often congregated in these relatively wet areas.

The three localities were visited a total of 38 times between October 7 and November 20, 1967. A set of 24 observations was made during each visit. The diel distribution of visits was: 5 visits between 0720 and 0913 hrs, 11 visits between 1019 and 1308 hrs, 3 visits between 1340 and 1515 hrs, 8 visits between 1526 and 1723 hrs, 8 visits between 1700 and 1825 hrs, 2 visits between 1820 and 1905 hrs, and one visit between 2020 and 2100 hrs by spotlight. Localities II and III were visited only five times each.

Fifteen physical and nine biological variables were measured to relate emergent clingfish with their environment (Table I): *locality,* scored 1–3; *date,* in days from the first visit; *time of day* (hrs), 0700–2100; *wave height* (m), from trough to crest of swells about 25 m offshore; *water level,* scored 1–8, from minimum low tide in calm sea to maximum high in rough sea; *dryness, upper rocks,* scored 1–3, from the condition when all surfaces of the three rocks were wet to that when the upper halves of rocks 1 and 2 and all of 3 appeared dry; *relative humidity* (%) measured with a sling psychrometer; *air* and *water temperatures* (°C), water measured in the surf with bucket thermometer; *wind direction,* scored 1–4—NW, WNW, W, SW—and *velocity* (mph), recorded as the average of three measurements by hand-held anemometer; *overcast,* scored 1–10, from clear and sunny to complete overcast; *sun on rocks,* scored 1–3, from total shade, through hazy sun, to bright sun; *irradiance* (langlies/hr), measured in shade or sun, depending on the condition of light on the rocks, with an integrating photometer (Haley, 1967); *barometric pressure* (millibars), recorded off station from a barometer in the laboratory.

We observed fish from a distance of about 20–30 m; closer approaches disturbed them. Recorded abundances were the averages of three counts, which included all visible fish along or above the upper margin of the seaweed fringe on the rock surfaces. Numbers of *fish* on *rocks 1–3,* recorded separately, were also summed to obtain *total fish*. Other observations were recorded for rock 1 only: the number of *fish* along the *seaweed fringe,* the per cent of *fish on* the *lower half of* the *rock* surface, the per cent of *fish with heads* pointed *down,* and the per cent of *juvenile fish* (young and halfgrown). The density of the *fish in crevices* and seaweed patches was scored 1–3 from scarce to abundant.

TABLE I

*Variables possibly affecting clingfish emerged in the rocky intertidal of central Chile**

Variable	Range	Mean	Standard deviation	Communality
Locality	1–3	1.29	0.61	0.56
Date	1–51	28.1	13.6	0.23
Time of day (hrs)	815–2100	1396.	354.	0.53
Wave height (m)	0.6–2.75	1.33	0.67	0.60
Water level (score)	1–8	4.82	1.89	0.71
Dryness, upper rocks (score)	1–3	1.84	0.73	0.83
Relative humidity (%)	52–96	73.3	12.0	0.61
Air temperature (°C)	11.5–18.3	15.7	2.30	0.78
Water temperature (°C)	11.3–14.7	12.6	0.91	0.42
Wind direction (score)	1–4	2.68	1.27	0.50
Wind velocity (mph)	1.2–17.3	5.93	3.96	0.47
Overcast (score)	1–10	4.39	3.65	0.48
Sun on rocks (score)	1–3	1.87	0.85	0.64
Irradiance (langlies)	0–3.3	1.55	0.83	0.64
Barometric pressure (mb)	29.79–30.11	29.93	0.10	0.48
Fish, total	0–135.0	35.1	31.6	0.99
Fish, rock 1	0–131.3	26.0	30.0	0.99
Fish, rock 2	0–23.7	4.92	5.59	0.78
Fish, rock 3	0–18.0	4.19	4.56	0.66
Fish, seaweed fringe	0–12.1	2.26	2.56	0.50
Fish on lower half of rock (%)	0–100	80.4	28.1	0.48
Fish with heads down (%)	0–80	30.4	22.8	0.57
Juvenile fish (%)	41–100	93.9	14.6	0.67
Fish in crevices, etc. (score)	1–3	1.97	0.71	0.11

* Each variable, expressed by 38 observations, in defined in the text. The communality (0–1) measures interactions with the other variables.

Factors were computed to assemble groups of interacting variables into a few causal arrays, each defining a system of interactions either within the environment or between clingfish and environment (*cf.*, Sokal and Daly, 1961). First a 24 by 24 matrix of correlation coefficients, one coefficient for each pair of variables, was "factored" to extract latent roots and orthogonal vectors of 24 principal factors. Then a much smaller number of factors was obliquely rotated to "simple structure," so that fewer factors defined more natural groups of variables because these factors were no longer necessarily orthogonal to each other; *i.e.,* they could be intercorrelated (*e.g.,* Cattell, 1965; Harman, 1967). In this way, "factor analysis represents covariation by finding fewer dimensions of variation than the number of variables in a correlation matrix" (Thomas, 1968: 849). Because we had no *a priori* way of estimating the number of natural groups in the system of 24 variables, we compared three different representations composed of three, four, and five rotated factors, respectively. The four-factor representation of the system appeared to be the most meaningful, because (1) two of the five factors were strongly intercorrelated (*cf.*, Thomas, 1968) and (2) three factors did not distinguish two important groups.

Each group was ordered into an array by decreasing magnitudes of the "loadings" of its several constituent variables on its factor (Table II). The loading of a variable was a measure of the variable's relative "importance" to, or correlation with, the factor, and was somewhat arbitrarily adjudged "significant" if it equaled or exceeded an absolute value of about 0.50 (Sokal and Daly, 1961).

Communalities estimated the proportion of the variation of each variable attributable to its covariation with the others (Cattell, 1965; Harman, 1967). Variables with high communalities interacted strongly with other variables. Variables with low communalities, however, explained relatively little of the system, in that a relatively large part of their variation was not attributable to their covariation with the others. This "error variance" was partitioned out of the analysis, so that it did not influence the factor loadings. The program BMDX72 for computation of the correlation matrix and communalities, extraction of 24 principal factors, and the subsequent rotation of a few factors to simple structure (Dixon, 1967) was modified for the IBM 360–75 computer at the University of California, Santa Barbara Computer Center.

Laboratory observations

Caught near localities I and II, experimental fish lived unfed in the laboratory in 40-liter plastic aquaria half-filled with aerated seawater of salinity about 34‰ at 12–17° C.

Responses of clingfish to aeration, stagnation, temperature, enforced emersion, and fresh water were noted of fish placed 1–3 at a time in covered 12-liter plastic aquaria. Seven of the fish were classified as adult (130–190 mm long), nine as halfgrown (100–120 mm), and two as young (60–90 mm). Submerged fish were observed in aerated seawater sun-warmed from 13 to 19° C or in seawater first aerated for 30 minutes, then allowed to stagnate in the shade. Emerged fish were observed on moist paper towels in an empty aquarium. In the two stagnating aquaria, body movements and opercular rates were recorded at 30-minute intervals during eight hours for three each of the halfgrown and adult fish as dissolved oxygen content, measured by galvanic-cell oxygen electrode, decreased from saturation. Six halfgrown and adult fish were subjected to serial dilutions of seawater.

Percentage concentrations of oxygen and carbon dioxide, by volume, of gas held in the gill cavities were measured after three fish, emerged for varying periods, had expelled it: an adult, 184 mm total length weighing 92.9 g; a halfgrown, 101 mm, 11.9 g; and a young, 67 mm, 3.3 g. Each fish voided bubbles through its gill slits while being gently submerged on a mechanical platform in a 12-liter aquarium half filled with a 1% solution of polyethylene oxide in seawater (Fig. 1). "Polyox" is a resin, dissolving slowly in seawater to form a non-toxic and viscous solution, which prevents the fish's ventilation and preserves any bubbles trapped in it (Todd, 1970). Expelled gas was caught in an inverted funnel filled with polyox and capped with a rubber diaphragm. Fish and funnel rested on a platform formed of a wire frame bent to two levels about one centimeter apart and surfaced with plastic screen. The platform was smoothly raised and lowered on a rack and pinion elevator from a photographic enlarger without disturbing the fish, which rested quietly on the upper level above the liquid while the inverted funnel waited on the lower level just below the surface.

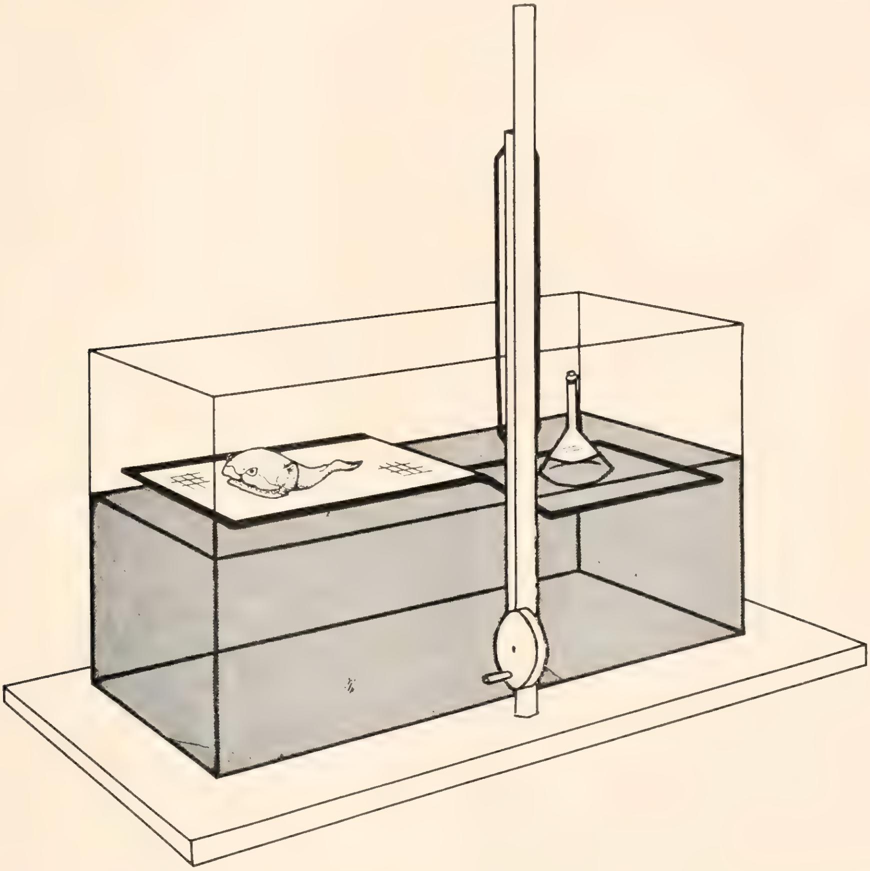

FIGURE 1. Experimental aquarium about half filled with a 1% solution of polyethylene oxide in seawater. An emerged Chilean clingfish rests above the surface on a split-level mechanical platform, which also supports an inverted funnel filled with the same "polyox" solution. Ending a specified period of emersion, the fish was slowly lowered into the liquid by cranking down the ratchet bar supporting the platform, so that the fish expelled bubbles out its gill slits into the polyox. The surfaced bubbles were preserved by the viscous polyox and captured in the funnel for later analysis of their gas content.

The fish remained quiet until slowly submerged at the end of each trial. Bubbles expelled from the gill slits were captured on the surface in the funnel, where they collected in the capped spout and were drawn into a one-milliliter syringe with dead space filled with saturated acid citrate solution. The gasping fish was quickly returned to the holding aquarium of aerated seawater, where it soon recovered for the next trial. For the adult and halfgrown, the volume of expelled gas was estimated in the syringe; for the young, in the capillary of a Scholander microgasometer, where the gas content was analyzed (Scholander, van Dam, Claff, and Kanwisher, 1955). Because gas was usually expelled in several bubbles from both gill slits, some was probably lost and most volumetric measurements were probably minimal. Little, if any, oxygen diffused through the polyox film. The known oxygen content (15–16%) of artificially reduced gas bubbled through polyox and collected after about 30 seconds was unchanged.

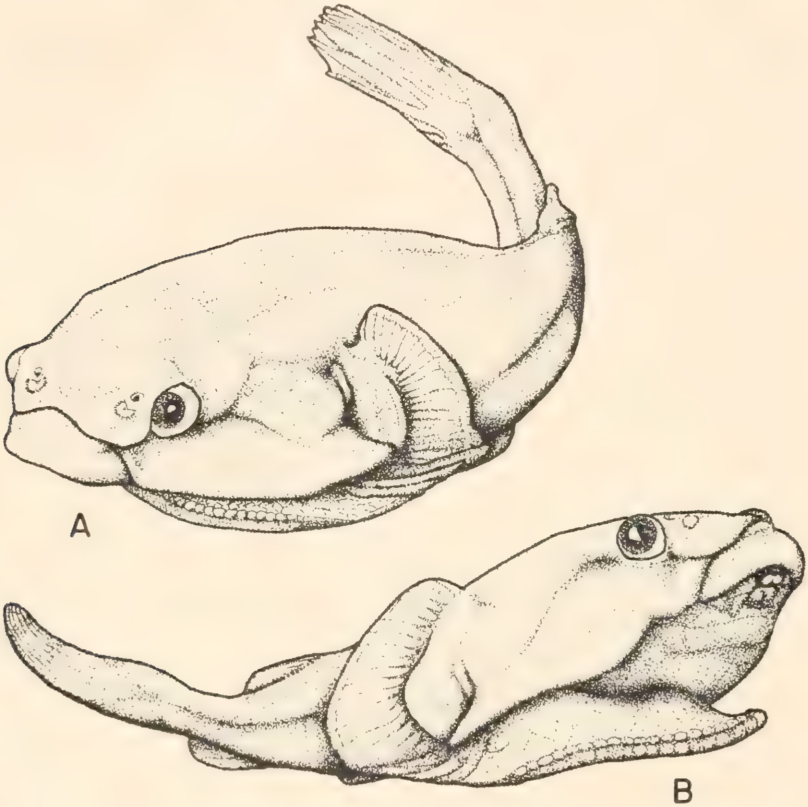

FIGURE 2. Clingfish emerged on a damp surface; A, flattened on the substrate to protect its delicate "frontal skin" on chin, leading edge of sucker, and pectoral fins; B, with head raised to expose this vasculose skin for cutaneous respiration.

Minimal rates of oxygen uptake were derived from oxygen depletion in the estimated volumes of branchial gas captured in the funnel, calculated as functions of presumed initial oxygen content (21%), content in the expelled gas, and the estimated total gas volumes (after Todd and Ebeling, 1966, where the brackets in the expression I $[(0.21 - P') - P]$ were inadvertently omitted). The initial volume (I) was taken as equal to or greater than the modal volume recorded for each fish. Volumes from the adult fish ranged from 1.4–2.5 ml, with the mode

2.2 ml. Therefore, 2.2 ml was substituted for all smaller measurements, which were probably of incomplete bubbles or bubbles emitted from one side only. All volumes exceeding 2.2 ml (2.3–2.5) were used because overestimations were unlikely. The "I" values for the halfgrown fish (0.09–0.85 ml, mode 0.20 ml) and young fish (0.01–0.04 ml, mode 0.03 ml) were estimated in the same way. Even though one value for the halfgrown fish was four times larger than the mode, it was retained as a possible measure of maximum gulping capacity.

Rates of oxygen uptake (milliliters per kilogram of wet body weight per hour) were calculated as the oxygen volumes multiplied by 1000 ÷ weight of fish (g), and by 60 ÷ period of emersion (min). Rates after the branchial gas was apparently renewed during a single trial were estimated as functions of the total duration of the trial, minus the time to renewal as indicated by a sharp rise in oxygen content in previous measurements made after certain intervals: adult fish, 57 min; halfgrown fish, 35 min; young fish, 12.5 min (Fig. 3).

Table II

Factors that group the variables in Table I into causal arrays*

Factor I: tranquillity		Factor III: abundance	
*_Wind velocity_	−0.75	*_Fish, rock 1_	0.99
Fish, rock 2	0.75	*_Fish, total_	0.97
Wave height	−0.68	_Juvenile fish_	−0.77
*_Fish, total_	0.58	_Fish, seaweed fringe_	0.68
Fish with heads down	0.55	_Locality_	0.54
*_Fish, rock 1_	0.46		
Factor II: water-level		**Factor IV: insolation**	
Dryness, upper rocks	−0.84	_Air temperature_	0.91
Water level	0.78	_Relative humidity_	−0.79
Fish, rock 3	0.78	_Water temperature_	0.72
Overcast	0.53	*_Irradiance_	0.67
Fish on lower half of rock	−0.53	_Wind direction_	0.48
*_Irradiance_	−0.48	*_Wind velocity_	0.48

* Variables are ordered by their relative "importance" as indicated by their loadings (numbers at right). Those marked by asterisk load "significantly" or nearly so on two factors, thereby indicating the factors' mutual interaction. Derivation of the factors is explained in the text.

Results

Effects of the natural environment

Communalities of the variables ordered the whole system into a hierarchy of links (Table I). The biological variables averaged higher communalities (0.71) than the physical (0.57) because they responded to several physical causes as well as interacting strongly among themselves. (The variable _fish in crevices_ was excluded from the averaging because it seemed to vary randomly.) The variables of fish abundance had high communalities because emergent fish responded to short-term changes in _water level, dryness of upper rocks,_ and _wave height,_ and tended to congregate in patterns. Physical variables like *date* and *water temperature,* on the other hand, had relatively low communalities because they probably involved long-term changes and, consequently, had little effect on the fish during the short study period. Water temperature, which varied but 3° C, may respond more to long-term fluctuations of the offshore current than to local heating during the Chilean spring. Even though they did not seem to affect fish abundances directly, the effects of insolation like *air temperature* and *irradiance* had fairly high communalities because they interacted strongly among themselves.

The factors divided the system into its component arrays of variables (Table II). The variable loadings identified the factors and revealed important environmental effects on the clingfish. The four factors were dominated by decreasing wind velocity and wave height ("tranquillity"), increasing water level with wetting of upper rocks ("water-level"), generally increasing fish abundance ("abundance"), and warming with decreasing humidity ("insolation"). For a given factor, physical variables should have the higher loadings if they evoke responses in the biological, but if biological loadings exceed physical, they may respond to other causes (Thomas, 1968). Physical variables of the tranquillity and water-level factors averaged higher loadings (0.72) than did the biological variables (0.64). Therefore, most fish came out onto wet rocks during calm periods when they often turned heads down and moved higher as the tide rose. The abundance factor, having no predominating physical loadings, simply indicated that more fish, especially adults, emerged in the more remote localities. The insolation factor, having no biological loadings, simply grouped the obvious effects of solar warming, which generated the land-sea breezes.

Several simple correlations apparently indicated indirect effects of the environment. Six variables did not load "significantly" on any factor; *i.e.,* they fell outside all four causal nexuses. *Fish in crevices, date,* and *barometric pressure* were apparently unimportant links within the system defined by the sampling regime. *Time of day, wind direction,* and *sun on rocks,* however, correlated significantly ($P < 0.05$) with several other variables in the insolation and abundance factors, which, in turn, interacted with the tranquillity factor (Table II). These simple correlations and the interactions between factors would seem to indicate that fish came out in the late afternoon when the surfaces of the observed rocks were in the sun, were it not for the multitude of fish counted during the single night station at Montemar. Perhaps more fish emerged at populous Montemar (where almost 75% of the stations were occupied) as people left the beaches in the late afternoon and, coincidentally, as sea breezes subsided and the sea calmed.

Emersion in aquaria

When halfgrown and adult clingfish emerged head first from aerated seawater, they immediately gulped air and perhaps water, stopped all opercular movement, closed their gill slits, and occasionally after several minutes turned heads down. Usually, however, they remained submerged or only partly emerged. Fish completely emerged and clinging to the smooth aquarium side gradually slipped back into the water until partly submerged. Adults placed on a horizontal platform relaxed immediately and soon appeared oblivious to laboratory activity, although emergent young moved their opercles and shifted position from time to time.

When submerged horizontally on a mechanical platform, all fish first fluttered their pectoral fins to break the suction through a groove in their ventral sucker, then moved their opercles to expel the bubbles through their gill slits.

Effects of temperature

Water warmer than 15° C disturbed submerged fish, which often clustered near the aquarium aerator and "panted" with strong and rapid opercular beats. When the seawater had warmed to an afternoon high of 17.8° C (room temperature, 20.1° C), most fish had pushed either their heads or tails above water. Those with heads submerged continued opercular beats, while those with heads out of the water had stopped all such movement.

Warm air disturbed emerged fish and seemed to elicit compensatory behavior because these fish stopped all stress reactions when subsequently cooled. An adult and halfgrown fish transferred from aerated seawater at 16.8° C to an empty aquarium in air 18.5° C closed their gill covers and were initially quiet. After a few minutes, however, the halfgrown fish began to "pant," apparently trying to pump moisture from the aquarium bottom through its gill cavities. The adult interspersed longer quiescent periods with shorter panting episodes for about an hour, then rested with gill covers closed while the halfgrown fish continued panting. At first, bubbles surrounded the opercles of both fish, as though the fish were trying to use their pharyngeal pumps. But when obviously stressed and drying fish were lightly sprinkled with seawater, they quickly relaxed and stopped panting. The halfgrown fish died within five hours with its opercles spread. The adult lived for nine hours, during which its skin became tacky. (In another trial, an adult survived 20 hours, although a halfgrown fish lived but 5 hours.)

Morphological changes accompanied the behavioral reactions to this temperature-induced respiratory stress. The pharyngeal epithelium of the emerged adult fish was reddish with vascularization and its dark red gills were engorged with blood. At one time the anterior holobranch adhered to the front of the gill chamber, while the others were clumped and pressed against the back. A thin membrane behind the chin closed the then cup-like chamber from beneath. In contrast with the pharynx, the mouth was pale to white. During active periods as the air temperature approached 18° C, the adult raised its head to expose a broad area of relatively delicate, vasculose, and unpigmented "frontal skin," extending from its chin over the broad front of its sucking disc to its pectoral fins. This area was previously hidden when the fish pressed flat to the substrate and exposed only its relatively thick, mucus-laden, and dark dorsal skin (Fig. 2A). When the fish reared its head, we saw that the frontal skin had been transformed from a white surface showing few capillaries to a reddish surface beset with conspicuous networks of engorged capillaries (Fig. 2B).

Effects of dissolved oxygen

Submerged clingfish showed no particular resistance to critically low concentrations of dissolved oxygen, although the fish usually emerged before it suffocated. In the two stagnating aquaria, adult and halfgrown fish behaved normally until the concentration fell below 2 ml/l, when all four fish had partly emerged and had increased their opercular rates by almost 70% (Table III). While oxygen measured more than 1 ml/l, fish usually clung to the aquarium wall tails up with their heads submerged and opercles moving. Then, as oxygen continued to fall, more and more partly emerged fish clung heads up and stopped their opercular movement. While emerging, they appeared to gasp as their noses broke the water's surface. (One halfgrown fish paused with nose barely protruding the surface, while it repeatedly gulped air possibly mixed with water.) Submerged fish became restless, then breathed rapidly and laboriously for varying periods before emerging.

TABLE III

*Responses of initially submerged clingfish to decreasing dissolved oxygen**

Dissolved oxygen concentration (ml/l)	Water temperature (°C)	Position of fish						Opercular rate (beats/min)	
		Adult			Halfgrown			Adult	Halfgrown
		E/T	Pe	H/E	E/T	Pe	H/E		
5.00–7.00	12.5–13.8 (13.0)	1/16	0.1	0/1	0/10	—	—	47–120 (82)	36–160 (105)
2.00–3.51	12.3–14.6 (13.3)	4/13	0.2	0/4	0/13	—	—	46–113 (82)	55–105 (80)
1.00–1.71	14.0–15.3 (14.6)	6/15	0.1–0.6 (0.35)	0/6	2/7	0.3–0.5 (0.38)	0/2	102–205 (144)	93–203 (148)
0.45–0.89	12.2–15.8 (14.5)	11/19	0.1–0.5 (0.38)	6/11	9/14	0.1–1.0 (0.44)	3/9	121–198 (140)	105–206 (152)

* Three adult and three halfgrown fish were observed in two aquaria that were first aerated, then allowed to stagnate. For columns under "position of fish," total observations (T) included the number of sightings of fish entirely or partly emerged (E) and the number entirely submerged (T-E). The observations of emerged fish (E) included the estimated proportions of their bodies above water (Pe) and the number of fish with heads uppermost (H), as compared with the number either lying parallel with the seawater surface or with tails uppermost (E-H). Ranges of observations precede averages in parentheses.

Survival of fish in deoxygenated seawater of less than 2 ml/l varied considerably. Although one adult fish died in 4 hours, the other survived 36 hours, including 24 hours in water containing less than 1 ml/l oxygen, as it mostly rested with parts of its body above water. One halfgrown fish survived three days in water aerated but once every 24 hours.

Effects of salinity

Clingfish showed a surprising tolerance of fresh water. Four adult and halfgrown fish lived submerged without apparent distress through a series of dilutions: 24 hours in 50% seawater, then 5 hours in 25% seawater, 5 hours in 12% seawater and 24 hours in pure tapwater. Another halfgrown fish transferred directly from 100% to the 12% seawater, however, turned pale and panted for about ten minutes before returning to normal. Only this halfgrown fish and another adult that was placed directly into tapwater died before the end of the experiment, some 58 hours after the first dilution.

Gas exchange in gulped air

Of the three clingfish emerged above the polyox solution, the adult appeared most quiescent and seemed to replenish the gas held in its gill cavities after the longest intervals. Soon after gulping air upon its removal from aerated sea-

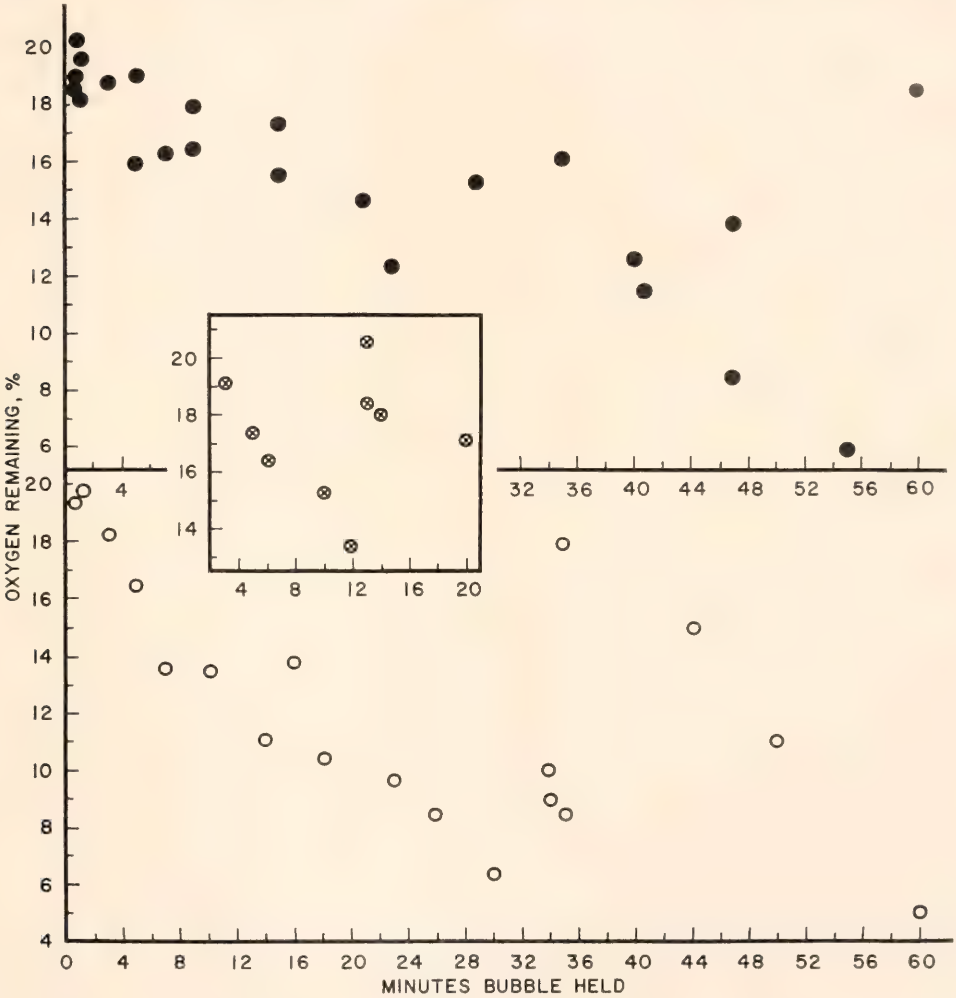

Figure 3. Temporal decrease in percentage oxygen in new or renewed gas held in the gill cavities of emerged clingfish; top (solid circles), expelled from a 93 g adult fish; bottom (open circles), from a 12 g halfgrown fish; inset (crossed circles) from a 3.3 g young fish. Each circle represents one measurement of the percentage oxygen in expelled gas after the fish had remained emerged on a mechanical platform for the time indicated on the horizontal axis.

water, it stopped all opercular and body movements and appeared undisturbed by activities in the laboratory. Percentage oxygen in its branchial gas expelled into the polyox solution decreased up to almost one hour, when the final observation indicated a sharp increase (Fig. 3, solid circles). This increase was unconfirmed because long experimental trials were difficult to complete. The struggling fish entering the polyox occasionally destroyed its expelled bubbles before they could be secured in the funnel, and the chance of disturbing the fish increased with time, so that reruns took up to four hours for one measurement.

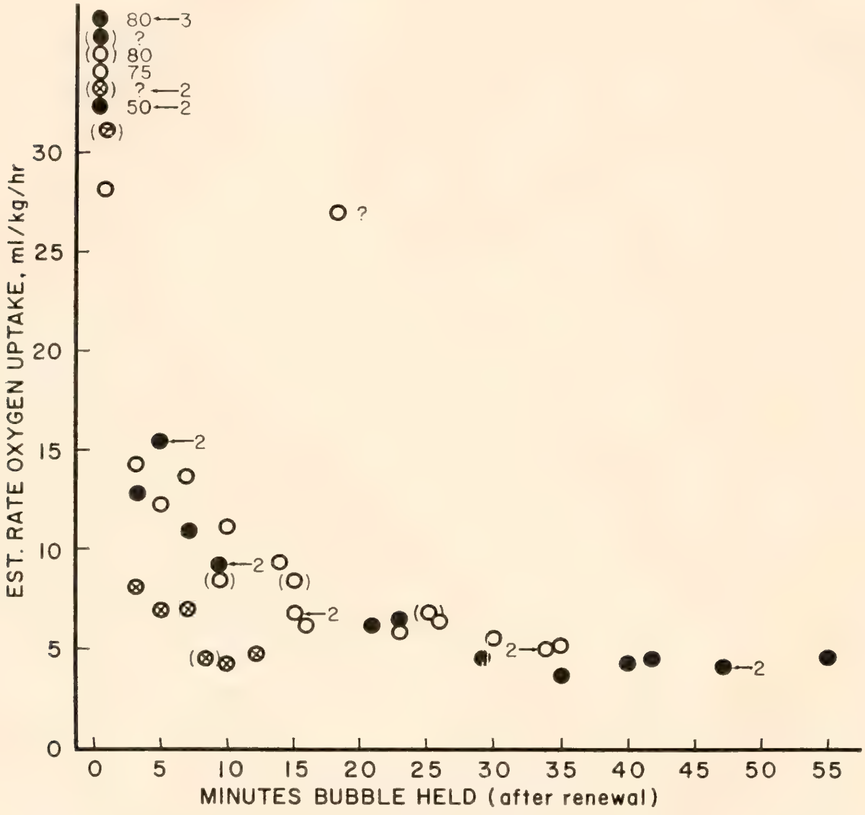

FIGURE 4. Temporal decrease in the estimated rate of oxygen uptake from gas held in the gill cavities of clingfish emerged in the experimental aquarium described in Figure 3; solid circles, adult fish; open circles, halfgrown fish, crossed circles, young fish. Parentheses enclose points depicting rates estimated from volumetric oxygen depletion after the gas had been once depleted and then renewed (see text). Circles at the upper left depict off-scale approximations of rates (numbers at right) measured after the fish had been emerged for relatively short periods of time. Problematical values are queried. Arrows indicate the average of two or three observations.

The quicker response of the halfgrown fish supported the tenuous hypothesis of gas renewal as indicated by the adult trials. Nineteen trials showed a similar sharp increase in oxygen content after slightly more than 30 minutes (Fig. 3, open circles). Because individual trials were shorter, a complete breathing cycle was observed, showing that oxygen decreased after renewal as before: within 35 minutes, the fish had gulped, depleted, expelled, and replenished its branchial air. Like the adult, the halfgrown fish quickly relaxed on the platform, where the oxygen content of its branchial gas reached a minimum of 4–6%.

The young fish was easily disturbed, occasionally moving its opercles and frequently expelling its branchial gas. The general pattern of oxygen depletion in this gas, however, resembled the others (Fig. 3, crossed circles). Trials lasting up to 12 minutes showed a steady decrease of oxygen to 13.4%, more than twice the minimum of the adult and halfgrown fish.

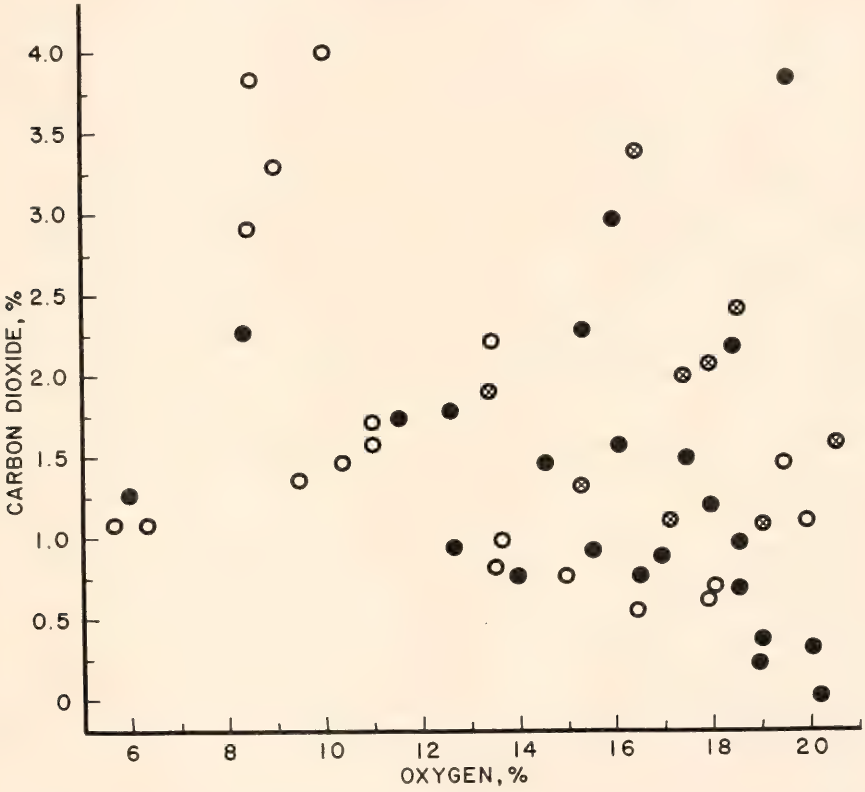

Figure 5. Increasing percentage carbon dioxide as a function of decreasing oxygen in gas held in the gill cavities of clingfish emerged in the experimental aquarium described in Figure 3; solid circles, adult fish; open circles, halfgrown fish, crossed circles, young fish.

All fish held about the same relative amount of oxygen in their branchial bubbles. To compare potential oxygen supplies, ratios of modal volumes of expelled gas to body weight were divided by hours to renewal and multiplied by 1000 to rid decimals. At first glance, the adult index (25) indicated that the adult fish carried about the same supply as the halfgrown fish (29), although both indices were considerably less than that for the young fish (45). Recall, however, that the young fish used but 36% of its oxygen, compared with 76% for the others. A corrected index of 23 (36/76 × 45) showed that the young fish actually held only slightly less oxygen for its size than the others.

Rates of oxygen uptake from the branchial gas decreased precipitiously during the first ten minutes of emersion, from an almost inestimable high to only 5–10 ml/kg/hr (Fig. 4). After the initial decrease, the adult rate averaged 5.3 ml/kg/hr, the halfgrown 6.8, and the young 6.2. (At about eight minutes, however, the rate of the young fish was only half that of the others.) During a 17-minute trial, the halfgrown rate was 27 ml/kg/hr (queried in Fig. 4), as calculated from an exceptionally large volume of expelled gas. Rates estimated for periods after gas renewal (points in parentheses) approximated the others.

The respiratory quotient of clingfish, as estimated from branchial gas exchange, was not unusual. Plots of carbon dioxide increase as a function of oxygen decrease, however, were scattered because the small observed percentages of carbon dioxide approached the experimental error and because several trials of less than five minutes yielded bubbles with unusually high percentages of carbon dioxide (1.5–3.8%), as though the gas were accrued near the onset of the trials (Fig. 5). The regression coefficient, which probably underestimated the respiratory quotient (*cf.*, Carter, 1957), was 0.23, compared with 0.28 for airbreathing mudsuckers (*Gillichthys mirabilis*), as determined by Todd and Ebeling (1966). The young clingfish expelled gas containing proportionately more carbon dioxide (avg. 1.8%) than either the halfgrown (1.5%) or adult fish (1.3%).

Discussion

Adaptations to optimal conditions

Factor analysis indicates that when the Chilean clingfish *Sicyases sanguineus* is in the field it avoids stress by its distribution and behavior. It positions itself so that it stays wet and relatively cool. It clings to exposed rocks continuously sprayed by the rough surf, which remains cool even on warm days. Fish even congregate on sunny surfaces so long as they are in a splash zone. The rise and fall of the water level apparently controls the vertical distribution of terrestrial fish, which cling to the upper rocks only when these rocks are splashed by high water. Beach crowds, however, may inhibit emersion of adults, even onto the preferred wet rocks with broad vertical surfaces.

Direct observations of clingfish both in the field and in the laboratory substantiate these conclusions drawn from factor analysis. In the field, fish were either restricted to the spray zone or to narrow cascades left by waves breaking over the rocks. During especially low tides, fish left the drying upper rocks. In general, they were either splashed or submerged by waves about once every five minutes and were never seen to wait longer than 15 minutes between wettings. Laboratory fish, which were not continuously splashed, often remained submerged or assumed a half-in and half-out position. Perhaps this is a compromise between total emersion without splashing, which would cause water loss and overheating, and continuous submersion, which could seem unnatural to the fish. Disturbed fish in relatively warm laboratory air appeared noticeably relieved when sprinkled with cool seawater. Like the mudskipper *Periophthalmus sobrinus* of eastern

Africa (Harms, 1935; Stebbins and Kalk, 1961), a terrestrial clingfish may normally not lose much water because it keeps its skin moist. Rao and Hora (1938) observed that a blenny, *Andamia heteroptera*, of southeastern Asia always lives above water in the intertidal spray zone, following the rising and falling tides.

It follows that the "insolation" factor does not directly affect clingfish because they avoid overheating as well as dehydration. They can markedly lighten or darken the mottled color pattern of their exposed skin, thereby controlling heat absorption. Fish held for several days in the relatively warm laboratory were generally lighter than recently captured fish, which, however, may have altered their color to blend with dark rocks in the intertidal. Stebbins and Kalk (1961) suggested that mudskippers may control their body temperature by changing skin color and avoiding midday heat. Gordon, Boëtius, Evans, McCarthy, and Oglesby (1969) presumed that for Nosy Bé mudskippers, which are protected by scales, overheating may be more hazardous than dehydration.

Survival during stress

A Chilean clingfish perhaps stranded during a violent winter storm could apparently survive substantial water loss on a drying rock, or substantial warming, stagnation, and dilution in an isolated tidal pool. Gordon, Fischer, and Tarifeño (in preparation) showed that fish survive substantial dehydration in the laboratory and Eger (in preparation) showed that other, smaller species of clingfishes tolerate surprising amounts of evaporative water loss. Even though a Chilean clingfish can survive several hours of aquatic anoxia by partial emersion, it is no more tolerant of low dissolved oxygen when it is submerged than most fishes (*cf.*, Jones, 1964). But like the Nosy Bé mudskipper *Periophthalmus sobrinus* (Gordon, Boëtius, Boëtius, Evans, McCarthy, and Oglesby, 1965) and the estuarine mudsucker *Gillichthys mirabilis* (Todd and Ebeling, 1966) it survives in very dilute seawater. In three months of searching during the relatively dry Chilean spring, however, we found only one fish isolated in an upper tidal pool, a tiny young individual. We saw none on dry rocks. Also, we counted relatively few fish on exposed rocks during the only rainy day of the field study. Although we found no fish in bays, sheltered inlets, or river mouths, Dr. Hugo Campos of the Universidad Austral (personal communication) reportedly collected a small young individual in the Río Valdivia, several kilometers from the sea.

Adaptation to airbreathing

Airbreathing in the Chilean clingfish may have originated as a means to survive stagnation in isolated tidal pools. In general, the ability of fish to live in deoxygenated water by breathing air at the surface may constitute a preadaptation to living on land (Carter, 1957; Saxena, 1963; Johansen, 1968). Although airbreathing is rare in most groups of marine fishes, it is relatively common among species of gobies, blennies, and clingfishes that inhabit estuaries or the intertidal zone (Schöttle, 1931; Oglialoro, 1947; Bertin, 1958; Saxena, 1963; Gordon, 1966; Eger, in preparation). And many of these species can live out of the water, escaping the intense competition and predation from the more abundant and diverse communities of subtidal predators.

The Chilean clingfish seems to have adapted behaviorally, morphologically, and physiologically to breathe air through its gills. In general, respiratory organs of amphibious teleosts include the skin, gills, mouth, pharynx, gut, and swimbladder (*e.g.*, Carter and Beadle, 1931; Carter, 1957; Krogh, 1959; Saxena, 1963; Johansen, Lenfant, Schmidt-Nielsen, and Petersen, 1968). The clingfish lacks a swimbladder and does not swallow air. Therefore, gills, skin, and buccopharyngeal epithelium are potential respiratory surfaces for emerged fish, which may consume oxygen more rapidly than submerged fish (Gordon, Fischer, and Tarifeño, in preparation). Although all three organs may contribute substantially to aerial respiration, the branchial organs seemed to be the most specialized. The tightly shut gill cavities of emerged fish may hold a mixture of air and water and serve as a kind of lung, protected from drying, sealed with liquid, and provided with a large lamellar surface covered with water for oxygen absorption. The branchial gas is not used for flotation because the fish, which lacks a swimbladder, expels the gas as it submerges and quickly dives to the bottom. The gas is not used as an oxygen store in stagnant water because it is always expelled. Vargas and Concha (1957a) observed that emerged fish with gills blocked by algin paste survive only about one-sixth as long as control fish.

Clingfish always expelled the spent gas through their small gill slits. Under optimal conditions, furthermore, the fish's head-down position on a vertical surface would facilitate release of gas upward through their watery branchial cavities. Most other airbreathing teleosts expel such gas through their gill slits rather than out their mouth (Todd and Ebeling, 1966; Johansen, 1966; Johansen, Lenfant, Schmidt-Nielsen, and Petersen, 1968).

Relatively few fishes breathe air through their gills, because gill lamellae tend to clump in air and because oxygen is usually absorbed through some epithelium aside from the gill membrane, where carbon dioxide is most easily eliminated (Carter, 1957; Krogh, 1959; Johansen, 1966). Of those that do, a freshwater knife fish of South America, *Hypopomus brevirostris*, gulps air at the surface (Carter and Beadle, 1931), while an eel, *Symbranchus marmoratus*, often emerges from stagnant swamp waters and even hides in terrestrial burrows (Johansen, 1966). Among estuarine fishes, the mudskipper of Australia *Periophthalmodon australis* reporterly has its gills modified for aerial respiration (*cf.* Berg and Steen, 1965) and *Periophthalmus sobrinus* in eastern Africa carries mixtures of air and water in its pharyngeal and gill cavities for aerial respiration and forcefully expels the spent gas in a spray of water from its gill slits (Stebbins and Kalk, 1961). Across the Mozambique Channel, however, the conspecific Nosy Bé mudskipper apparently does not have to do this in order to breathe on land (Gordon, Boëtius, McCarthy, and Oglesby, 1969). Eger (in preparation) observed that a small amphibious clingfish of the Gulf of California, *Tomacodon humeralis,* holds bubbles of air in its moist gill cavities when it comes out of the water. Rao and Hora (1938) concluded that the terrestrial blenny *Andamia heteroptera* breathes air held in its tightly shut gill cavities.

The gills of Chilean clingfish are structurally adapted for airbreathing, more so in adult than in young fish (Vargas and Concha, 1957a). Adult fish have fewer lamellae per millimeter of gill filament and have more widely spaced lamellae on the exposed distal half of the filament than do young fish, which seem generally less well-adapted to terrestriality. Gordon, Fischer, and Tarifeño (in preparation) noted that young fish do not survive enforced emersion as well as do adults, and the present study indicated that emergent young are much less composed than adult fish. In general, teleostean fishes that have proportionately smaller gill surfaces may survive better in air because their lamellae are more dispersed (*e.g.,* Schöttle, 1931; Gray, 1954). Indeed, all amphibious fishes that reportedly breathe air through their gills have lamellae that are widely spaced or otherwise structurally modified to prevent clumping and collapsing in air (Carter and Beadle, 1931; Schöttle, 1931; Johansen, 1966).

Clingfish out of water in laboratory aquaria waited surprisingly long times between breaths, while most of the oxygen was being depleted from the air held in their closed gill cavities. The adult fish waited for almost one hour, about twice as long as the halfgrown fish and four times as long as the young fish. Although the two larger fish had used up almost 80% of their oxygen, the young fish, occasionally moving its opercles between breaths, had used only about 40%. Johansen (1966) observed that *Symbranchus marmoratus* gulps air at the surface of deoxygenated water at varying intervals of time, averaging about 15–20 minutes while it uses up about 50% of its branchial oxygen, but occasionally extending to 30 or 40 minutes while it uses some 80% of its oxygen. The obligatory airbreathing electric eel *Electrophorus electricus* ascends at least once every two minutes (Johansen, Lenfant, Schmidt-Nielsen, and Petersen, 1968). Out of the water, the European eel *Anguilla vulgaris* inflates its gill cavities with air, which it regularly renews about once a minute at room temperature (Berg and Steen, 1966). Precht (1939) showed that cycles of airbreathing vary considerably in freshwater pulmonate snails, which may use up as much as 99% of their oxygen between breaths.

Emerged clingfish slow their heart rate and breathe at varying rates (Gordon, Fischer, and Tarifeño, in preparation). Submerged birds and mammals show a general diving syndrome including bradycardia, which prevents asphyxia and shunts oxygenated blood under pressure to the vital organs (Scholander, 1940; Andersen, 1966). Obligatory water-breathing teleosts respond in a similar way when they are emerged (Leivestad, Andersen, and Scholander, 1957; Garey, 1962). The Australian mudskipper *Periophthalmodon australis,* on the other hand, is so well adapted to life out of water that its heart beat actually slows when it is submerged, as though aquatic life were completely foreign to this fish (Garey, 1962). Like the Chilean clingfish, however, a few other amphibious fishes show some tendency toward a "diving syndrome" in air, although usually less so than typical water-dwelling fishes (Berg and Steen, 1965; Todd, 1970). In the African mudskipper *Periophthalmus sobrinus,* the response is not detectable (Gordon, Boëtius, Evans, McCarthy, and Oglesby, 1969) and in the eel *Symbranchus,* the heart rate increases after an initial period of bradycardia. Perhaps handling of the experimental clingfish intensified their bradycardia and thereby slowed their oxygen consumption (*cf.,* Kisch, 1950; Leivestad, Andersen, and Scholander, 1957), although emerged mudsuckers (*Gillichthys*) slow their heart rate markedly even with their brain removed (Todd, 1970), and mudskippers actually increase their heart rate when disturbed (Gordon, Boëtius, Evans, McCarthy, and Oglesby, 1969).

The metabolic requirements of clingfish in air may differ from those of fish in water. They breathe sporadically in air, sometimes at a faster rate than they do in water, but apparently slow down or stop breathing altogether at respiratory plateaus (Gordon, Fischer, and Tarifeño, in preparation): young fish out of water in moist respiratory chambers at room temperature consume oxygen at rates of 50–110 ml/kg/hr after 1–3 hours out of water and at 120–180 ml/kg/hr after 11–13 hours out of water; 30–80 gram halfgrown and adult fish at 2–93 (avg. 32) ml/kg/hr during 0–12 hours out of water and 4–44 (avg. 18) ml/kg/hr during 15–23 hours out of water; and aquatic young and adult fish more regularly at 32–50 (avg. 40) ml/kg/hr. Vargas and Concha (1957a) noted that the average rate of oxygen uptake at room temperature for a 120 g airbreathing adult fish, 46 ml/kg/hr, is low, compared with rates of many aquatic teleosts. It approximates the standard rate of the European eel breathing air for four hours (Berg and Steen, 1965) and of the adult mudsucker (Barlow, 1961), but approaches only half the rate of the Nosy Bé mudskipper (Gordon, Boëtius, Evans, and Oglesby, 1968). Although the European eel decreases its rate of oxygen uptake after several hours in air (Berg and Steen, 1965), *Symbranchus* actually increases its oxygen uptake in air (Johansen, 1966). Todd (1970) inferred that the mudsucker *Gillichthys* in an anoxic atmosphere can decrease its metabolism to almost zero before it finally suffocates in comparative peace.

The branchial oxygen of clingfish may sustain their lowest rates of aerial oxygen consumption, but only 12–30% of their average rate, assuming no replacement of the gas between breaths. Gordon, Fischer, and Tarifeño (in preparation), furthermore, questioned the need of fish to close their gill cavities when out of the water in their natural habitat, where they are regularly splashed by cold water. Emerged *Symbranchus marmoratus* keeps its single gill slit tightly closed for about 30 minutes, but then becomes agitated and opens its mouth to facilitate gas exchange (Johansen, 1966). When *Gillichthys mirabilis* comes out of the water, it moves its slightly opened mouth as it holds a bubble of air in its buccopharynx (Todd, 1968). Perhaps clingfish are more active and aware in the field than they are in the laboratory, and so replenish their branchial air more frequently in the intertidal splash zone. But they may have difficulty exposing their small mouth, which is inferior in position and, therefore, is usually pressed against the rock surface.

Terrestrial clingfish may also breathe through their skin. Krogh (1904) demonstrated that European eels with gills blocked can consume about 60% of their normal aquatic oxygen requirement through their skin. Berg and Steen (1965) concluded that eels consume only about one third of their total oxygen through inflated gill cavities and the rest through their skin (augmented briefly by swimbladder oxygen). Terrestrial mudskippers breathe cutaneously as well as branchially, perhaps balancing pathways without changing overall rates (Gordon, Boëtius, Evans, McCarthy, and Oglesby, 1969), and occasionally consuming some 60% of their total oxygen through their skin (Teal and Carey, 1967).

Clingfish probably breathe through their relatively delicate and vasculose "frontal skin," because the tough, thick, dark, and mucus-laden dorsal skin protecting most of their body and fins appears unsuitable for oxygen absorption (at least in adults). Fish often raise their head to expose their frontal skin, which is shaded when they cling with head pointed downward. Other amphibious teleosts have potential respiratory epithelium located near the front of their body. Mahajan (1964) observed that the lips, barbels, and ventral adhesive pad of the Indian catfish *Glyptothorax telchitta* redden conspicuously as the emerged fish gasps air, which is forced out through its gill cavities. Vessels in the pectoral fins of airbreathing mudsuckers become noticeably engorged with blood (Todd, 1970).

Contact with air apparently stimulates clingfish to gulp. When they emerged snout first, they gulped air immediately, then closed their opercles, but when they emerged tail first, they continued their opercular movements so long as their head remained under water. Also, fish in stagnating water did not necessarily emerge and occasionally remained "panting" under water until they suffocated. Stebbins and Kalk (1961) observed that emerging mudskippers of eastern Africa quickly expand their gill cavities, then tightly close their small gill slits to secure air and water for branchial respiration on land, although Gordon, Boëtius, Evans, and Oglesby (1968) found no water in the buccopharynx and gill cavities of emerging Nosy Bé mudskippers, even though they made similar gulping movements. Johansen (1966) concluded that the most effective way to stimulate airbreathing in *Symbranchus* is simply to drain its aquarium, a much more effective stimulant than either enforced hypoxia or hypercarbia.

Unlike the Chilean clingfish, airbreathing fishes that inhabit stagnant waters of tropical swamps and coastal estuaries gulp air when they encounter varying degrees of aquatic hypoxia (*e.g.*, Todd and Ebeling, 1966; Johansen, Lenfant, and Grigg, 1967; Johansen, 1968). Both aquatic and atmospheric hypoxia stimulate the European eel to breathe air (Berg and Steen, 1965, 1966), although only atmospheric hypoxia so stimulates the obligatory airbreathing electric eel, which does not normally come out of the water (Johansen, Lenfant, Schmidt-Nielsen, and Petersen, 1968). Willmer (1934), however, inferred that environmental concentrations of both dissolved oxygen and carbon dioxide interact to control respiration in a freshwater characid of South America, *Erythrinus erythrinus*, which breathes air through its vasculose and physostomous swimbladder. Also, Precht (1939) showed that dual effects of hypoxia and hypercarbia are additive as they stimulate and control ventilation of freshwater pulmonate snails. Various vasomotor and pressure responses may control blood flow through the respiratory organs of airbreathing fishes (*cf.*, Steen and Kruysse, 1964; Berg and Steen, 1966; Johansen, 1968; Todd, 1970).

Relatively large proportions of carbon dioxide in the gill cavities of airbreathing clingfish did not noticeably affect their ventilation or general behavior. Concentrations as large as 4 vol. % were measured in bubbles of gas expelled from fish forcefully submerged in laboratory aquaria. Nevertheless, young fish, whose expelled gas contained proportionately more carbon dioxide than that of adult fish, were more active and easily disturbed when out of the water. Also, as emerged adults dried off, they became distressed and finally opened their gill cavities, raised their head, and panted. They seldom opened their small mouth, however, when they raised their head to expose their frontal skin.

Terrestrial fishes that are subject to drying may not be able to eliminate access carbon dioxide from closed respiratory organs. Johansen (1966) pointed out that such fishes risk severe hypercarbia, because the normal pathway for eliminating carbon dioxide through their gills is blocked. During enforced periods of emersion, in fact, *Symbranchus marmoratus* becomes agitated and opens its mouth after about 30 minutes. Airbreathing European eels, on the other hand, may eliminate most of their excess carbon dioxide through their moist and vasculose skin (Krogh, 1904). Gas containing more than 5 vol. % carbon dioxide passed directly over the gills of emerged eels inhibits the depth and frequency of their ventilation, although similar gas mixtures passed over their skin have no such effect (Berg and Steen, 1965). Constantly wetted by ocean spray, therefore, Chilean clingfish in the field should easily eliminate excess carbon dioxide through their skin, even while their gill cavities are tightly closed. The branchial organs of fish moistened in the laboratory showed an expectedly low respiratory quotient.

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Mr. R. Ibara kindly assisted Ebeling with all computations and completed the factor analysis at the U.C.S.B. Computer Center during Ebeling's sabbatical leave provided by the University of California.

SUMMARY

The amphibious clingfish *Sicyases sanguineus* attaches by means of its ventral sucker to vertical surfaces of large exposed rocks splashed by the cool and heavy surf of Chile and southern Perú. Although adult and halfgrown fish tolerate diluted seawater and can survive at least a few hours in warm and stagnant water, they seldom, if ever, occur in isolated tidal pools, bays, or estuaries.

Factor analysis indicates that clingfish come out of the water more abundantly during periods of calm and often turn head-down. They avoid drying rocks outside the spray zone and emerge onto higher rocks as the water level rises. Adult fish come out of the water more abundantly in remote areas relatively undisturbed by civilization. Insolation apparently does not directly alter the abundance of clingfish, which act so as to minimize evaporative water loss and overheating.

Terrestrial fish breathe air held in their gill cavities, probably through their gills. As they come out of the water, fish gulp air, then stop all opercular movements to seal their cavities, and often turn head-down. This positioning may facilitate airbreathing by easing the expulsion of spent gas upward through the watery gill cavities and by shading an area of delicate respiratory epithelium under

the chin. The volume percentage oxygen in gas expelled into a viscous solution of resin in sea water decreased regularly for about 12 minutes in a young fish, about 30 minutes in a halfgrown fish, and about one hour in an adult before these fish renewed their branchial gas. Although a concomitant increase in percentage carbon dioxide indicated that the branchial gas contributed to respiration, rates of oxygen uptake calculated from modal volumes of expelled gas were only about 12–30% of the fish's total long-term rate in air as determined by other investigators. As in other airbreathing fishes, however, cutaneous respiration may supplement branchial respiration, which fills the total need only when the metabolic rate falls.

Exposure of their head apparently stimulates clingfish to gulp, so that atmospheric air, rather than aquatic hypoxia, is their primary stimulation to airbreathing. Fish in stagnating water do not necessarily emerge and occasionally remain under water until they suffocate. Like other airbreathing fishes, clingfish appear insensitive to relatively large proportions of carbon dioxide in their branchial gas. And even with their opercles closed and their ventral mouths pressed against the substrate, clingfish in the field should easily eliminate excess carbon dioxide through their wet skin.

Vargas and Concha (1957a) emphasized the ontogenesis of terrestriality in clingfish from erratic young to well-regulated adult: modification of gill surface to minimize clumping, control of aerial oxygen uptake, and greater composure on land. The present study indicates that exposure of an anteroventral respiratory membrane, assumption of a head-down position, improvement of gulping technique, control and slowing of ventilation, and greater efficiency of exchange between branchial gas and blood also contribute critically to the maturing fish's increasing independence of aquatic life.

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THE EVALUATION OF THE "*CALLIPHORA* TEST" AS AN ASSAY FOR ECDYSONE[1]

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Thirty-five years ago one of us (Fraenkel, 1935) showed that abdomens of a fly larva, *Calliphora erythrocephala*, which had been isolated by ligation before a critical period and normally would never pupariate, could be induced to tan by injection of hemolymph from pupariating larvae. This experiment was confirmed on *Calliphora* (Becker and Plagge, 1939) and *Didacus ciliatus* (Narayanan and Lai, 1954). It subsequently became the method, now commonly known as the "*Calliphora* test," by which ecdysone was isolated, and with little modification is still the only practical method for identifying and assaying ecdysone. The validity of the original experiment was recently cast in doubt by Ohtaki, Milkman and Williams (1968) who failed to obtain pupariation by the injection of hemolymph in a member of a different family, *Sarcophaga peregrina*. They also used for their assays animals prepared in an entirely different manner, and on the basis of their data considered the 1935 experiment an erroneous observation, based on the appearance of "false positives." They stated that the entire larva at the critical period contained only 7% of a *Sarcophaga* unit of ecdysone, and quoted estimates of the ecdysone titre in *C. erythrocephala* by Shaaya and Karlson (1965) in support of this contention. A more recent analysis of the distribution of ecdysone in different tissues of *C. erythrocephala* by Shaaya (1969) was also in agreement with these views.

We have now reproduced the original results in *C. erythrocephala* and a related species, *Phormia regina*, as well as in *Sarcophaga argyrostoma*. Further probing into the conditions under which tanning occurs during puparium formation of flies led to the discovery of a neurohormonal effect which accelerates the onset of tanning (Zdarek and Fraenkel, 1969). This observation suggested that under natural conditions, the amount of ecdysone required for tanning might be smaller than what had hitherto been determined by the standard test as a *Calliphora* (or *Musca* or *Sarcophaga*) unit.

These considerations further led to an analysis of the various factors which affect the outcome of the traditional *Calliphora* test in four different species of flies: *Calliphora erythrocephala*, *Phormia regina*, *Sarcophaga bullata*, and *S. argyrostoma*.

Some preliminary remarks about the terminology concerning the *Calliphora* test for ecdysone are in place. Not only has the test been, and will be increasingly used with other fly species, such as *S. peregrina* (Oktaki, Milkman and Williams, 1967), *S. bullata*, *S. argyrostoma*, and *P. regina* (this paper), *Musca domestica*

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(Kaplanis, Tabor, Thompson, Robbins and Shortino, 1966; Adelung and Karlson, 1969), but several steroids of the general structure of ecdysone have been employed. We shall refer to the test as the "pupariation test for ecdysone," speak of ecdysone(s) when referring to the whole group of compounds, and use the terms α-ecdysone and β-ecdysone (rather than the synonyma ecdysterone, crustecdysone, 20-hydroxyecdysone) when referring to a particular compound. Instead of using "*Calliphora* unit" as a general term we shall refer to the particular fly species to which a particular determination applies, or use the general term "pupariation unit of ecdysone."

Materials and Methods

Species of flies used

Specimens of *Calliphora erythrocephala* Meigen, and *Phormia regina* L. (both Calliphoridae), *Sarcophaga bullata* Parker and *S. argyrostoma* Robineau-Desvoidy (Sarcophagidae) were used.

Breeding techniques

In much of the work reported below we used a modification of the culture technique which was suggested to us by Dr. W. L. Downes, Michigan State University. The feeding fly larvae with their food (pork liver) are loosely wrapped in a sheet of aluminum foil and placed on top of a layer of sawdust. Larvae which have finished feeding leave the food and descend into the sawdust. By removing larvae from the sawdust at regular intervals, groups of larvae are obtained of much more homogeneous physiological age than when keeping the whole batch together.

Ligation

In the standard tests larvae were ligated at a time when about 10–20% had already pupariated. Ligatures, using cotton thread, were placed well behind the ganglionic mass, approximately behind the 5th visible segment, at a distance of about one-third of the length of the larva counting from the front.

Injection

Hind sections of ligated larvae were injected by means of finely drawn glass pipettes with a diameter of about 40 μ at the tip, manufactured with the aid of a Micropipette Puller M1 (Industrial Science Associates, Inc., Ridgewood, New York). Pressure was applied by mouth via a piece of rubber tubing fixed to the pipette. Amounts injected were estimated by comparison with a calibrated length of the same tubing. Since the hind parts of larvae ligated in this position are paralyzed, no further immobilization is required. Solutions of ecdysone in distilled water were injected in volumes of 5 μl or less per test abdomen.

Scoring

The degree of tanning produced in response to a particular injection was estimated by a method similar to that developed by the Karlson group (Karlson and

Shaaya, 1964; Adelung and Karlson, 1969) and others. These authors score complete, marked, half, and no pupariation as 100%, 75%, 50% and 0%, with a 25% score added in the latest paper. We use the same principle, with the difference, that all partial pupariation was scored as 50%. Our procedure yields essentially the same percentage figures of pupariation as that obtained by other authors.

The ecdysone used throughout was β-ecdysone (obtained from Syntex Corp., Palo Alto, California). The reason for using β- rather than α-ecdysone were: (1) Its availability. (2) Demonstration that this form is the one present in fly larvae (Galbraith, Horn, Thompson, Neufeld and Hackney, 1969). (3) Demonstration that β-ecdysone is more active in *Sarcophaga peregrina* than α-ecdysone (Ohtaki, Milkman and Williams, 1967).

EXPERIMENTS

I. *The effect of ligation on pupariation in the front and hind parts of the larvae of four different fly species*

When a mature fly larva, with the crop empty, is ligated into two parts behind its ganglionic mass the anterior or posterior parts may or may not pupariate. It is generally known that if the ligature is made before a "critical period" only the anterior parts pupariate whereas the posterior parts remain untanned unless supplied with molting hormone (ecdysone). If ligated after the critical period, both sections tan, though the anterior part does so about two hours before the posterior part (Zdarek and Fraenkel, 1969). One would expect the anterior part to pupariate under all circumstances, since it contains the ring gland, the source of ecdysone. This is, however, not the case. The ligature seems to affect pupariation in the front part in various ways. Even when ligated after the critical period (when the hind part tans) the anterior part may fail to pupariate (for reasons never satisfactorily explained). This we consider an inhibition. We must expect similar incidents of inhibition also to occur in precritically ligated larvae. Ligation can also cause a delay in pupariation of the front part.

The foregoing remarks apply to all cyclorrhaphous flies, there are however, as will be presently shown, important specific differences in detail which have to be considered when drawing conclusions from one species to another. To this end we have timed the occurrence of pupariation in the front and hind parts of *C. erythrocephala*, *P. regina*, *Sarcophaga bullata* and *S. argyrostoma* after ligation and in relation to pupariation of unligated controls. The larvae were ligated when about 10% had already pupariated.

The reason why we have selected the 48 hours mark for discussing the results, and why the observations were not continued for much longer was the growing incidence of mortality after this period, and the increasing difficulty of discerning between anterior parts which are either dead, dying, or else no longer able to pupariate. The results are recorded in Figure 1, A–D.

In all four species the hind parts which pupariate do so about two hours after the front parts. (Cases where the posterior parts alone pupariated are excluded). No pupariation occurred in the hind parts later than 15 hours after ligaturing in *Calliphora* and *Phormia*, and later than 20 hours in the *Sarcophaga* species. The occurrence of "false positives" after a waiting period of 24 hours has not been

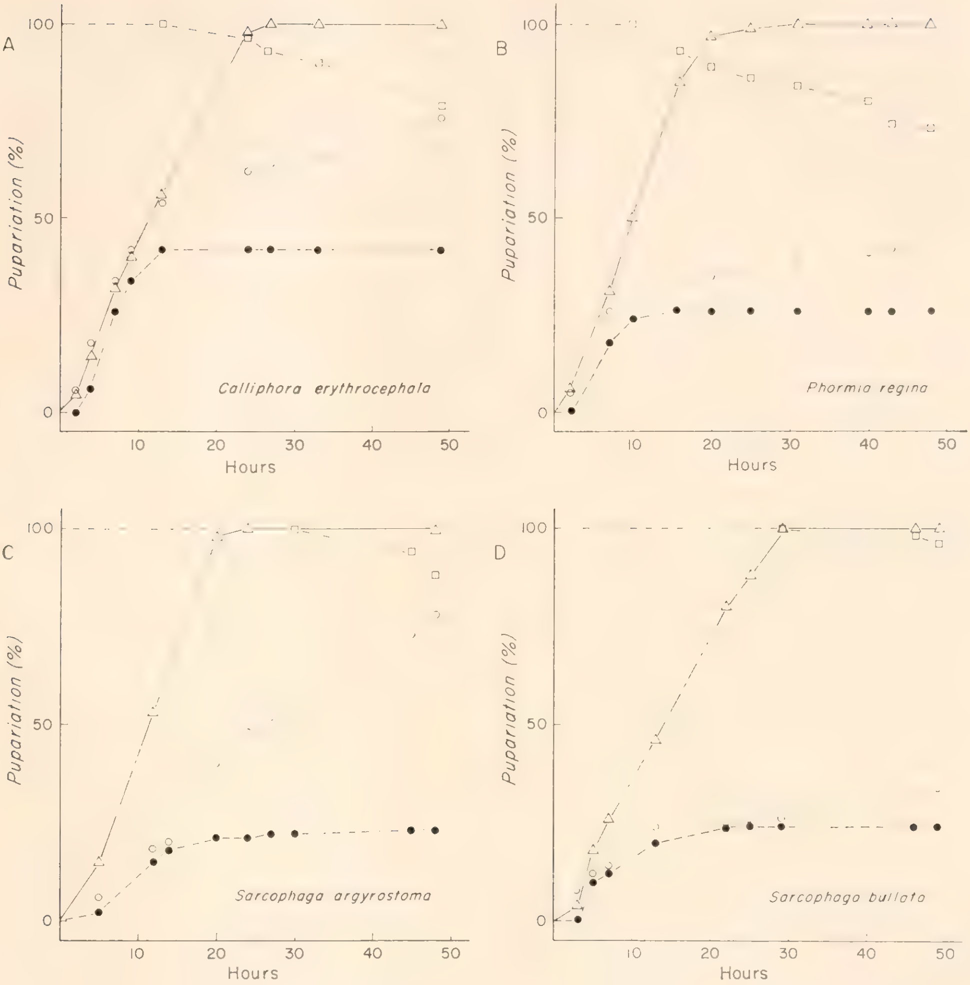

FIGURE 1. These graphs depict the course of pupariation in four species of flies in unligated controls △——△, the front parts ○·····○ and hind parts ●---● of ligated mature larvae. The top line □–•–•–□ represents mortality. A. *Calliphora erythrocephala.* B. *Phormia regina.* C. *Sarcophaga argyrostoma.* D. *Sarcophaga bullata.*

recorded. This eliminates the need for a further waiting period. Pupariation in the anterior parts in relation to that in the non-ligated controls does not seem to be delayed to any significant degree during the first 5 to 10 hours after ligating, but such a delay becomes very considerable in precritically ligated specimens, and is more marked in *P. regina* and *S. bullata,* than *C. erythrocephala* and *S. argyrostoma.*

In addition to this delay there is also an inhibition whereby many anterior parts fail to pupariate altogether. The incidence of this inhibition was very different in the different species. The proportion of precritically ligated larvae which by

48 hours after ligating had pupariated in the anterior part was 12% in *S. bullata,* 23% in *P. regina,* 59% in *C. erythrocephala* and 71% in *Sarcophaga argyrostoma.* The total percentage of larvae which had not pupariated in the front part after 48 hours was 65% for *S. bullata,* 57% for *P. regina,* 23% for *C. erythrocephala,* and 22% for *S. argyrostoma.* These figures are not the final results because more pupariation occurred at a later date. The count was terminated at this date for reasons stated above. Despite this uncertainty, the different trend in the different species is very clear.

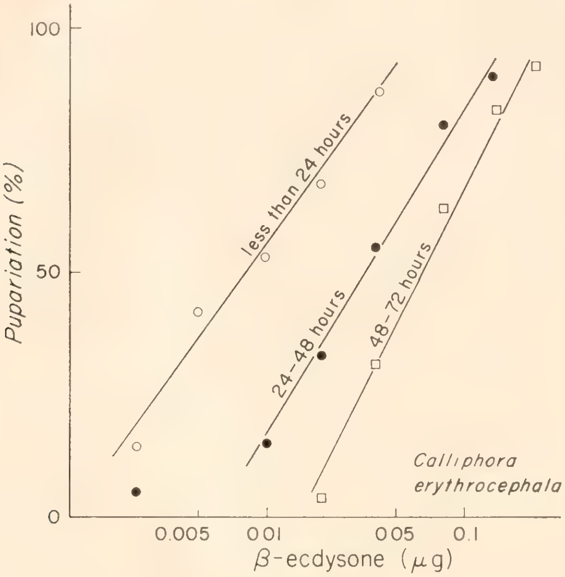

FIGURE 2. *Calliphora erythrocephala.* The effect of the injection of graded doses of β-ecdysone on pupariation in test abdomens from larvae where the anterior part alone had pupariated within 24 hours, between 24 and 48 hours, and between 48 and 72 hours after ligation.

It is interesting that in *S. bullata* and *P. regina,* where the delaying and inhibitory effect on pupariation in the precritically ligated larvae is stronger than in *C. erythrocephala* and *S. argyrostoma,* ligation after the critical period gave rise to many more specimens where the hind part alone pupariated (*S. bullata* 16%, *P. regina,* 45%, *S. argyrostoma* 4%, and *C. erythrocephala* 3%).

Since the first prerequisite for a successful ecdysone test is as high a yield as possible of specimens which pupariate in the anterior part alone within the first 24 hours after ligating, *S. bullata* and *P. regina* are far less suitable test subjects than *C. erythrocephala* and *S. argyrostoma.*

II. *Variability of the pupariation unit of ecdysone*

Karlson and Hanser (1953), Karlson and Shaaya (1964), and Adelung and Karlson (1969) have previously shown that the amount of ecdysone necessary to induce pupariation in the ligatured hind part is very much dependent on the physiological age of the preparation at the time of injection. This age effect should be considered from two different perspectives. One is the presumed age of the larva in relation to pupariation which, in view of the large individual variation in any one batch can only be guessed from the time of pupariation in the controls, and from the period between ligation and pupariation in the front part. Since, as seen in Figure 1, pupariation in the normal controls was about completed after 24 hours, we may assume that all anterior parts which are tanned by that time had done so at more or less their appropriate time. All pupariation subsequently is increasingly delayed. The second consideration concerns the time interval between pupariation in the front part and the actual injection.

TABLE I

Calliphora erythrocephala, *effect of delay of injection of ecdysone on pupariation of test abdomens. β-Ecdysone was injected at different periods into test abdomens of larvae in which the anterior part had pupariated within 24 hours after ligaturing. Each horizontal line represents tests performed simultaneously on larvae of the same batch, n = number of test abdomens per test, % = score of pupariation.*

β-Ecdysone per test abdomen (μg)	Score of pupariation in test abdomens injected at the following periods after ligaturing					
	24 hours		48 hours		72 hours	
	n	%	n	%	n	%
0.006	12	42	11	32		
	12	41	10	30		
0.012	11	82	11	64		
	11	45	10	40		
0.025	11	86	11	64		
	12	54	10	50		
0.05	9	94	8	87		
0.1	11	82	12	63	9	50
0.2	11	95	12	87	10	60

1. *Injection into test abdomens of different physiological ages.* Mature larvae of *Calliphora erythrocephala* were ligated, and the larvae which subsequently pupariated in only the front section divided into three groups according to the interval between ligation and pupariation: (1) Within 24 hours; (2) Between 24 and 48 hours; (3) Between 48 and 72 hours after ligation. Group 1 was injected 24 hours, group 2, 48 hours, group 3, 72 hours after ligation. Responses to injection of each particular dose of ecdysone were estimated on the basis of three assays in group 1 and 2, and 2 assays in group 3, comprising 10 to 15 specimens at each assay. It was necessary to make several replications for each dose to draw the straight lines of Figure 2, since response to a particular dose varied considerably when larvae from different batches were used. This, by itself, shows that the bioassay is not very well reproducible.

The results (Fig. 2) show that test abdomens of group 1 were greatly more sensitive than of group 2, with those of group 3 still less sensitive. The value of the *Calliphora* unit, as defined above, was 0.008, 0.035, and 0.07 μg β-ecdysone per test abdomen for groups 1, 2, and 3, respectively.

2. *Injection into test abdomens of the same physiological age after different waiting periods.* The posterior parts of larvae whose anterior part alone had pupariated up to 24 hours after ligating were injected with graded doses of β-ecdysone at 24, 48, and 72 hours after ligating. The result (Table I) shows a lower sensitivity of test abdomens to injected β-ecdysone when the test abdomens had been further "aged." The differences between the 24 and 48 hours injections are significant at the 0.004 level (two tailed), by the Wilcoxon paired-sample rank test.

3. *The dilution effect.* In the pupariation test for ecdysone various authors injected the hormone solutions in portions of 10 (*Calliphora, Sarcophaga*) or 5 μl (*Musca*) (see Discussion). This amount of solvent represents a far from negligible addition to the blood volume of the test abdomens. In order to examine the effect of such a dilution on pupariation β-ecdysone was injected into test abdomens of *C. erythrocephala* in volumes of 1 and 10 μl water. Table II shows that the pupariation score was consistently reduced with the higher volumes of injection.

TABLE II

Calliphora erythrocephala, *effect of the volume of injection on the pupariation score of test abdomens injected with ecdysone. Injections were made 24 hours or 48 hours after ligation (the anterior parts had pupariated before 24 hours or between 24 and 48 hours after ligation, respectively).*

"Age" of test abdomens hrs	β-Ecdysone (μg) per test abdomen	Score of pupariation			
		Injected in 1 μl		Injected in 10 μl	
		n	%	n	%
24	0.01	15	60	15	40
24	0.01	12	58	12	33
48	0.02	12	37	12	18

4. *The effect of a brain hormone on the pupariation unit of ecdysone.* In a preceding paper (Zdarek and Fraenkel, 1969) we had shown that the addition of an extract from neurosecretory cells in the brain not only accelerated the onset of pupariation but also increased the sensitivity of the test abdomens to injected ecdysone in *Phormia regina.* We have now investigated this phenomenon in greater detail in *C. erythrocephala.*

Precritically ligated larvae were divided into three groups according to the time when the anterior parts alone had pupariated: A. within 24 hours, B. between 24 and 48 hours, C. between 48 and 72 hours after ligation. The hind parts were injected with graded doses of β-ecdysone dissolved in water, and with a homogenate of CNS of *S. bullata* larvae from which the ring glands had previously been removed (Table III). Test abdomens of group A required less ecdysone for a given pupariation effect when also injected with the CNS extract. In applying the Wilcoxon paired-sample rank test, the percentages given in Table 3A were recalculated to the first place of decimals to resolve the ties between the first and fifth experiment. The differences in pupariation score in the presence or absence of CNS extracts were significant at the 0.04 level, two-tailed. Pupariation

TABLE III

Calliphora erythrocephala, *effect of graded dose of β-ecdysone on pupariation of test abdomens ligated at different physiological ages, in the presence or absence of homogenates from brains of* Sarcophaga bullata.

Injected per test abdomen		Pupariation score in absence of brain homogenate		Pupariation score in presence of brain hormone			Difference of percentage
vol. µl	β-ecdysone µg	n	%	brain equivalent*	n	%	
A. Anterior parts pupariated within 24 hours after ligation; injected 24 hours after ligation.							
5	0.004	10	35	1.5	13	27	−8
5	0.008	17	32	1.5	16	47	+15
10	0.01	9	17	2.0	10	55	+38
5	0.016	16	59	1.5	16	66	+7
10	0.02	14	46	2.0	14	54	+8
5	0.032	16	81	1.5	16	91	+10
10	0.04	14	61	2.0	14	79	+18
B. Anterior parts pupariated 24 to 48 hours after ligation; injected 48 hours after ligation.							
5	0.004	11	27	1.5	17	6	−21
5	0.008	12	29	1.5	17	3	−26
5	0.016	11	54	1.5	17	9	−45
5	0.032	16	84	1.5	15	57	−27
10	0.05	12	21	2.0	11	45	+24
10	0.1	12	58	2.0	11	72	+14
7	0.05	12	58	1.5	12	42	−16
7	0.1	12	67	1.5	12	71	+4
7	0.2	12	100	1.5	12	75	−25
C. Anterior parts pupariated 48 to 72 hours after ligation; injected 72 hours after ligation.							
10	0.1	11	36	2.0	11	18	−18
10	0.2	11	68	2.0	11	32	−36

* Number of brains per abdomen.

was also considerably accelerated in the presence of the CNS extracts. The larvae of group B and C which had been ligated at a younger age did not show either of these effects. Those of group C seemed to show an opposite effect of decrease of ecdysone action in the presence of the neurohormone.

III. *Pupariation induced in test abdomens of different fly species by the injection of hemolymph*

In order to test abdomens at their most sensitive state (see above) most injections were made 24 hours or earlier after ligaturing. Twenty-four hours after the injection the number and degree of pupariation, as well as the number of dead specimens, were recorded. The surviving untanned specimens which appeared in a sufficiently healthy state were then injected a second time and again scored after 24 hours.

The results are shown in Table IV. The final column, giving the total ratio, sums up the results of the first and second injection with the percentages calculated from the number of survivors after the first injection.

TABLE IV

Puparium formation in test abdomens of larvae of Calliphora erythrocephala, Phormia regina, Sarcophaga bullata *and* S. argyrostoma *as a result of injecting them with hemolymph from different donors at different times after the critical period.*

Test abdomens, species, time of injection after ligation	Hemolymph injected		Pupariation 24 hrs after				Total pupariation score	
	amount/test abdomen	Donor	1st injection		2nd injection*			
			n	%	n	%	n	%
C. erythrocephala								
24 hrs	10 µl	*C. erythr.*, white puparia	26	35	14	11	26	40
24 hrs	10 µl	*C. erythr.*, feeding larvae	38	3	32	6	38	8
24 hrs	10 µl	*S. bullata*, white puparia	17	0	8	0	17	0
24 hrs	10 µl	*S. argyr.*, white puparia	15	37	5	20	15	43
48 hrs	10 µl	*C. erythr.*, white puparia	35	0	27	20	35	16
48 hrs	10 µl	*C. erythr.*, feeding larvae	27	0	22	0	27	0
48 hrs	10 µl	*S. bullata*, white puparia	25	0	22	0	25	0
P. regina								
16 hrs	5 µl	*P. regina*, white puparia	24	12	16	16	24	23
16 hrs	5 µl	*P. regina*, feeding larvae	15	0	14	0	15	0
12 hrs	8–10 µl	*P. regina*, white puparia	23	22	16	25	23	39
12 hrs	8 10 µl	Ringer sol.	10	10	9	0	10	10
S. bullata								
24 hrs	10–15 µl	*S. bullata*, white puparia	30	2	26	2	30	3
24 hrs	10–15 µl	*S. bullata*, feeding larvae	24	6	19	0	24	6
24 hrs	15 µl	*C. erythr.*, white puparia	23	4	22	2	23	7
24 hrs	15 µl	*C. erythr.*, feeding larvae	22	0	22	0	22	0
S. argystoma								
24 hrs	15 µl	*S. argyr.*, white puparia	32	2	31	2	32	3
24 hrs	25 µl	*S. argyr.*, white puparia	16	28	11	14	16	37
24 hrs	15 µl	*C. erythr.*, white puparia	15	0	—	—	—	—
24 hrs	25 µl	*C. erythr.*, white puparia	20	40	9	22	20	45
24 hrs	10 µl	*S. argyr.*, concentrate**	15	17	—	—	—	—
24 hrs	25 µl	*S. argyr.*, concentrate**	12	67	—	—	—	—
24 hrs	25 µl	Ringer sol.	18	6	—	—	—	—
24 hrs	—	nothing injected	26	4	—	—	—	—

*Second injection into surviving larvae, 24 hours after first.
** Filtrate from heat-coagulated blood from white puparia concentrated 5 times.

There were conspicuous differences between the four species. *Calliphora, Phormia,* and *S. argyrostoma* yielded pupariation scores around 40%, figures similar to those previously reported, but only when tested under the most favorable circumstances. It was essential to control the time and dosage of injection. *Calliphora* responded when injected 24 hours, but not 48 hours after ligation. *S. argyrostoma* responded well to injection of 25 µl of its own hemolymph, but not at all to that of 15 μl. It responded equally well to 25 μl but not at all to 15 μl of *Calliphora* hemolymph. Reversely, *Calliphora* responded to 10 μl of hemolymph from *S. argyrostoma*, but not to that from *S. bullata*. This would suggest that the activity of the hemolymph of *S. argyrostoma* is higher than that of *S. bullata*. *S. bullata* showed no response to 10–15 μl of its own hemolymph or that of *Calliphora*. No further tests were undertaken with this species in view of the difficulty of obtaining suitable test abdomens (see above).

Active hemolymph was taken throughout from white puparia which, according to Shaaya and Karlson (1965) and Shaaya (1969), are at the peak of ecdysone activity. Hemolymph from younger, still feeding larvae (*i.e.*, at least a day before the critical period) was in all cases inactive, thus proving the specificity of the test.

In view of the larger size of *Sarcophaga* it is not surprising that a larger volume of injected hemolymph is required for pupariation. An attempt was made of preparing concentrates of hemolymph for the purpose of increasing its ecdysone content. Hemolymph was diluted with 3 parts of water, heated at 90° for 10 minutes, centrifuged, and the supernatent concentrated on the vacuum evaporator at 70° C to one-fifth of the original concentration. Ten microliters of this concentrate evoked a weak but clear tanning response, while 25 μl led to 67% pupariation. A comparison of the effects of 25 μl of the original blood and 10 μl of the concentrates shows that some activity was lost in the course of preparation of the concentrate.

DISCUSSION

It has recently been several times reported that the ecdysone titre in the hemolymph of larvae of cyclorrhaphous flies prior and during pupariation is far lower than the amount of exogenous hormone necessary to cause pupariation in the pupariation test for ecdysone (Shaaya and Karlson, 1965; Ohtaki, Milkman and Williams, 1968; Shaaya, 1969). Yet, we (this paper) and others (Fraenkel, 1935; Becker and Plagge, 1939; Narayanan and Lai, 1954) have shown that the hemolymph of pupariating larvae is capable to induce tanning in the pre-critically ligated abdomens. In order to solve this paradoxical situation we analyzed the pupariation test in great detail and revealed a number of factors which can affect the outcome of the traditional test.

1. *Age of the larvae at the time of ligation*

We (Fig. 2), as similarly before Karlson and Hanser (1953), Karlson and Shaaya (1964), and Adelung and Karlson (1969), have shown that test abdomens become greatly more sensitive to ecdysone nearer the time of pupariation.

2. *Waiting period between ligation and injection*

The test abdomen loses in responsiveness if injection of ecdysone is delayed (Table I). In Ohtaki, Milkman and Williams (1968) standard test abdomens a waiting period of 40 hours increased the *Sarcophaga* unit from slightly over 0.01 to about 0.035 μg. Loss of sensitivity by post-ligation aging was also recorded by Karlson and Hanser (1953). Waiting periods of 24 hours or longer were routinely employed by all previous authors.

3. *A dilution effect*

When ecdysone is injected into test abdomens of *Calliphora* (Adelung and Karlson, 1969) or *Sarcophaga* (Ohtaki, Milkman and Williams, 1967) in portions of 10 μl of an aqueous solution, or dissolved in 10% isopropanol, respectively, or of 5 μl in *Musca* (Adelung and Karlson, 1969), the hemolymph of the receivers becomes appreciably diluted (the blood volume of a *Calliphora* test abdomen is 20 to 25 μl). The effect which this overall change in concentration (not only of ecdysone) may have on tanning does not seem to have been considered. It clearly leads to an overestimate of the pupariation unit of ecdysone (Table II). Such effects are also noticeable in the data of Table III.

4. *The effect of a neurohormone*

Our previous data (Zdarek and Fraenkel, 1969) from *P. regina* had suggested that a humoral factor from the brain which accelerated the onset of pupariation also increased the sensitivity of the test abdomens to injected ecdysone. The same effect has now been shown in *C. erythrocephala*, but only in relatively mature test abdomens (Table III). A similar "synergistic" effect of a combined action of a brain hormone and ecdysone, both prepared from *Bombyx*, in the pupariation of *C. erythrocephala* had been discovered by Kobayashi and Burdette (1961). Thus, the absence of the neurohormone, as is the case in the standard test for ecdysone, may increase the requirements for ecdysone.

In all our experiments where injection of hemolymph induced pupariation, test abdomens of the highest possible sensitivity were employed, *i.e.* ligated as closely as possible before the critical period, and injected without a further waiting period with the highest possible amount of hemolymph. Thus, in the light of the various factors enumerated above—age, dilution effect, presence of neurohormone—the true *Calliphora* unit of ecdysone under the most favorable conditions may be substantially lower than the figure now given for the synthetic hormone (0.01 μg/test abdomen). Furthermore, the hemolymph of pupariating larvae contains, besides the humoral factors, a potent substrate-enzyme system, which may not be without a significance for the result of the test. Our results with interspecific transfusion of hemolymph confirm not only the non-specificity of its activity (Becker and Plagge, 1939), but also suggest that this activity can vary in different species.

Ohtaki, Milkman and Williams (1968) in explanation of their data with *Sarcophaga peregrina* suggest a mechanism by which ecdysone acted by a gradual accumulation of covert effects in response to subthreshold doses, rather than by massive accumulation in the hemolymph. In a current investigation on *S. argyrostoma*, to be published shortly, we have obtained direct experimental evidence in support of this contention, namely, that the effects of both exogenous and endogenous ecdysone can and do summate over a period of time. In the light of these findings it may well be that the amount of hormone contained in injected hemolymph which induces tanning in test abdomens is only the last quantity necessary to bring about the qualitative change—tanning—in test abdomens already preconditioned (sensitized) by subthreshold doses of the hormone. Then the final effect is the results of two factors, sensitivity of the test abdomen and activity of the hemolymph.

In considering the significance of the pupariation test for ecdysone we must realize that the *Calliphora* (*Sarcophaga*, *Musca*) unit of ecdysone can only refer to the particular and peculiar conditions of the test abdomens, and may bear little relation to the real hormone titre in the normal larva at the time of pupariation which is also affected by the rates of inactivation (Ohtaki, Milkman and Williams, 1968; Karlson and Bode, 1969; Shaaya, 1969) and continuous release of ecdysone.

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SUMMARY

1. The effect of ligation on pupariation in the front or hind parts of larvae of four species of flies, *Calliphora erythrocephala*, *Phormia regina*, *Sarcophaga bullata*, and *S. argyrostoma* was investigated. Ligation causes effects of delay or inhibition of pupariation which are very differently expressed in the four species. A large proportion of pre- or postcritically ligated specimens of *P. regina* and *S. bullata* altogether fail to pupariate in the anterior part. This makes these species unsuitable test subjects for the pupariation test for ecdysone.

2. Test abdomens of *C. erythrocephala* required significantly less ecdysone for a given pupariation effect when also injected with a CNS-extract. Tanning was also considerably accelerated in this case.

3. The value of the pupariation unit of ecdysone is influenced by a number of factors, such as age at the time of ligation, the waiting period between ligation and injection, the dilution effect of the solvent, and the simultaneous action of a neurohormone. The requirements for natural ecdysone in normal larvae at the time of pupariation are probably substantially lower than the values which have been determined by others with test abdomens and the use of synthetic ecdysones.

4. In confirmation of older data, and contrary to recent claims, tanning was induced in test abdomens of the larvae of *C. erythrocephala*, *P. regina*, and *S. argyrostoma* by the injection of hemolymph from pupariating larvae. *Calliphora* blood induced tanning in specimens of *S. argyrostoma*, and vice versa. The conclusions are drawn that differences between the different species in the action of ecdysone are of a quantitative rather than qualitative nature.

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CALCIFICATION IN ECHINODERMS: EFFECTS OF TEMPERATURE AND DIAMOX ON INCORPORATION OF CALCIUM-45 *IN VITRO* BY REGENERATING SPINES OF *STRONGYLOCENTROTUS PURPURATUS*[1]

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Studies of the calcareous endoskeleton of echinoderms have been reviewed by Hyman (1955), Raup (1966), Swan (1966), Nicol (1967), and Nichols and Currey (1968). Recent work on the echinoderm endoskeleton has been carried out by numerous investigators including Ebert (1967, 1968), Towe (1967), Travis, Francois, Bonar, and Glimcher (1967), Donnay and Pawson (1969), Kobayashi and Taki (1969), Märkel and Titschack (1969), Nissen (1969), Pilkington (1969), Weber (1969), and Weber, Greer, Voight, White, and Roy (1969). To date, emphasis has been placed on various aspects of skeleton composition, morphology, crystallography, growth and regeneration. However, no quantitative studies have been reported on the physiology of skeleton formation in echinoderms (see Robertson, 1941; Nichols, 1964; and Nicol, 1967).

Due to the absence of quantitative studies on calcification in echinoderms, calcified tissues of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson), were investigated with the objective of developing a method to study calcification rates under different conditions. Gradually, attention was focused on the spines. Preliminary experiments showed that intact spines incorporate variable and relatively low quantities of calcium-45. But, when spines were broken, rapid uptake of the label occurred as part of the process of skeleton regeneration. This observation suggested that regenerating spines might be useful as a calcifying system, and subsequent experiments confirmed this possibility.

Reported here are the results of experiments establishing regenerating spines of *S. purpuratus* as a useful tool to study calcification rates *in vitro* under different conditions with calcium-45 as a tracer. Using this calcifying system, the effects of temperature and a carbonic anhydrase inhibitor, Diamox (acetazolamide), on calcification rates are measured quantitatively.

MATERIALS AND METHODS

Animals

Adult specimens of *S. purpuratus*, ranging in wet weight from 40 to 70 grams, were collected subtidally at Flat Rock Point, Los Angeles County, California,

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and maintained in the laboratory under constant room lighting in filtered, recirculating sea water at 14° C. Several species of brown algae were supplied as food.

Experimental procedures

Aboral, primary interambulacral spines of similar size were fractured with scissors 2 to 3 millimeters above the milled ring. As a result of this procedure, the stubs were protected from damage during regeneration by the surrounding forest of intact spines about 10 millimeters longer. Usually, 5 to 8 spines were fractured on each of the 10 plate rows of the interambulacra, which yielded from 50 to 80 stubs per urchin depending on animal size.

Fractured spines were incubated *in vivo* or *in vitro*. In the latter case, the stubs were removed from the urchin by severing the tissue attaching the base to the underlying tubercle. Incubations were carried out under constant room lighting at 15° C in covered, plastic dishes with a capacity of 500 or 1000 milliliters (Stoway utility dish, Southern California Plastic Co., Glendale). Calcium-45 with a specfic activity of 19.9 mc/mg was obtained from New England Nuclear, Boston, Massachusetts. In all experiments, aeration of the incubation medium was achieved by means of water-saturated air bubbled through an air stone. In some of the experiments, stubs were incubated *in vitro* simultaneously with stubs *in vivo,* but in separate compartments within the incubation chamber to prevent damage to them by the activities of the animal. The compartments were constructed from halves of plastic, histological coverslip boxes fastened to the inside of the chamber with methylene chloride just below the water level. To estimate ^{45}Ca incorporated into the skeleton by exchange, control spines previously bleached for several hours in 5.25% NaOCl (commercial strength Purex or Clorox) were fractured as above and added to the incubation medium in some of the experiments. Further details of each experiment are given in the Results section.

After incubation, stubs were sampled, bleached in Purex for 2 to 3 hours or longer to remove soft tissue and unincorporated ^{45}Ca, rinsed several times in distilled water adjusted to pH 7.0, and oven dried at 110° C on filter paper. Between rinsings, stubs were drained briefly on filtered paper. Care was taken to protect the delicate regenerated mineral from damage during handling. Dried stubs were mounted in modeling clay and stored until the activity of ^{45}Ca was determined.

Assay of ^{45}Ca activity

Individual stubs were fixed vertically with modeling clay on planchets. The tip of the regenerated portion of the stub was then positioned 1 mm below the center of a thin end-window Geiger-Müller tube (LND Corp., Oceanside, New York, #733 T) with the aid of an adjustable platform which permitted reproducible counting geometry. Activity was determined with a transistorized scaler (Nuclear Supplies Inc., Encino, California, Model SA-250). After incubation in ^{45}Ca for several days, some radioactivity was detectable on the periphery of the spine shaft in addition to that of the regenerated tip. Radioactivity on the shaft was reduced to background levels during counting by slipping a rubber disc with a small hole in the middle over the spine tip to a position just below the level of fracture, care being taken not to touch the regenerated skeleton. The disc, of the same diameter as the planchet, was cut from the cuff of a rubber autopsy glove and pierced with a hot pin. Spines of different diameter were shielded by discs with holes of appropriate size. Each spine was counted for three minutes and the activity expressed as counts per minute per spine. In some of the experiments, activity of ^{45}Ca in the incubation medium and coelomic fluid was determined in triplicate, 50 microliter aliquots which were dried on planchets and counted for three minutes. This activity was expressed as counts per minute per milliliter. All counts were corrected for background.

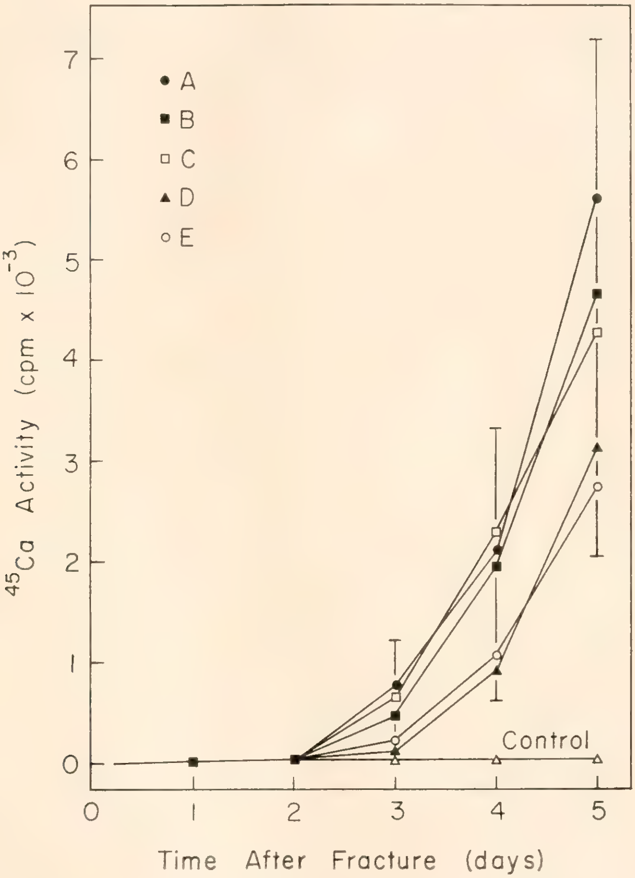

FIGURE 1. Kinetics of ^{45}Ca incorporation by regenerating spines of five specimens of *S. purpuratus* (A–E) incubated *in vivo* for five days following experimental fracture. Each point represents the mean value of 3 or 5 spines, with vertical bars indicating the range of all values. Control values are means of 3 bleached spines.

In experiments on the effects of temperature and Diamox on calcification rates, activity was determined independently by liquid scintillation techniques using the sample preparation method of Carr and Parsons (1962), adapted here to the assay of spines labeled with ^{45}Ca. Scintillation counting eliminated error due to self absorption. Each stub was inverted over a liquid scintillation vial and the tip snipped off with scissors about 1 millimeter below the level of experimental fracture. This procedure insured complete recovery of all labeled mineral deposited on the fractured surface during regeneration. In some of the stubs employed in experiments on the effect of temperature on calcification rates, the second millimeter of the shaft was also removed and placed in a separate vial to estimate the activity of ^{45}Ca in the first millimeter which was included with the regenerated mineral as a consequence of the sampling procedure. Two milliliters of 0.5 *N* HCl were added to each vial to dissolve the calcareous fragments. The vials were then gradually heated to about 130° C to remove water and HCl. After cooling, 6 milliliters of a mixture of toluene and ethanol (5:1), and 5 g/L of 2,5-diphenyloxazole (PPO), were added to each vial. Activity of ^{45}Ca was determined at room temperature in a Beckman liquid scintillation counter, Model LS-233. Counts

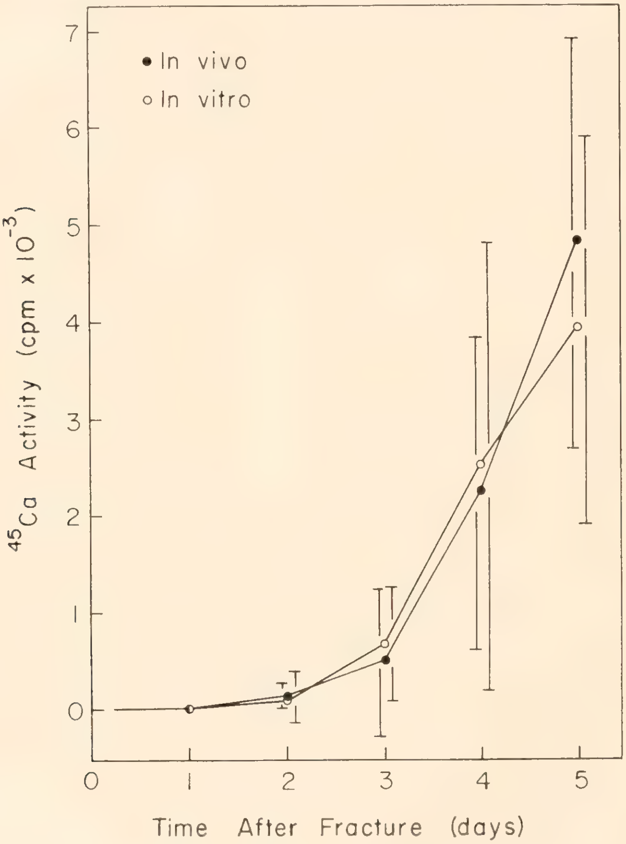

FIGURE 2. Kinetics of ^{45}Ca incorporation by regenerating spines of a single *S. purpuratus* incubated *in vivo* or *in vitro* for five days following experimental fracture. Each point represents the mean value of 5 spines, with vertical bars (left, *in vivo;* right, *in vitro*) indicating ± S.D.

were taken for five minutes, corrected for background, and expressed as counts per minute per spine. Error due to measurement of radioactivity was usually less than ± 5%.

Results

Comparison of calcification rates in vivo *and* in vitro

To determine when calcification was initiated during regeneration *in vivo,* five urchins with fractured spines were incubated individually in about 350 milliliters of sea water containing 1.0 μc/ml of ^{45}Ca. At daily intervals for five days, 3 or 5 stubs were removed from each animal and assayed for radioactivity.

Figure 1 shows that there was a lag period of about two days following fracture before ^{45}Ca was incorporated. During this time, the amount of label incorporated by regenerating spines was not significantly different from that incorporated in

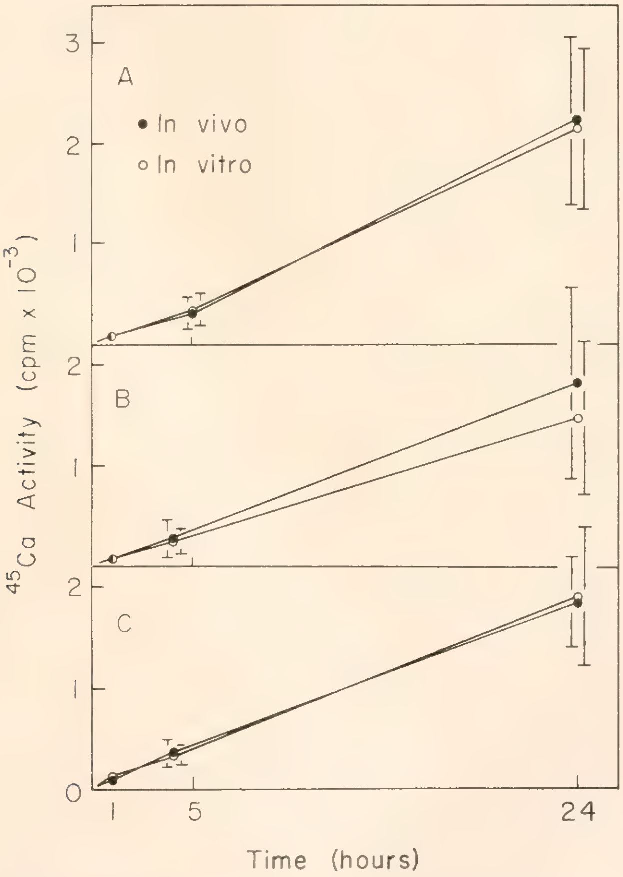

FIGURE 3. Kinetics of ^{45}Ca incorporation by spines of three specimens of *S. purpuratus* (A–C) incubated for 24 hours *in vivo* or *in vitro* after regeneration for four days *in vivo* in plain sea water following fracture. Each point represents the mean value of 10 spines, with vertical bars (left, *in vivo;* right, *in vitro*) indicating ± S.D.

bleached controls by exchange. Thereafter, uptake of ^{45}Ca by regenerating spines increased rapidly, with exchange accounting for only about 1% of the total activity incorporated four to five days after fracture.

During the first 24 hours' incubation *in vivo* there was a relatively rapid disappearance of ^{45}Ca activity from the incubation medium followed by a more gradual, nearly linear rate of removal. At the end of the incubation period, nearly 20% of the initial radioactivity was removed. In another experiment, the disappearance of radioactivity from the medium during the first 24 hours' incubation was concomitant with a rapid appearance of radioactivity in the coelomic fluid, which reached equilibrium with the medium after 30 to 36 hours' incubation.

To determine whether calcification of regenerating spines is initiated *in vitro*, fifty spines were fractured on a single urchin. Twenty-five stubs were then removed (explanted) and incubated *in vitro* simultaneously with the remainder *in vivo* in about 700 milliliters of sea water containing 1.0 μc/ml of ^{45}Ca. Five stubs were sampled daily from each group for five days and assayed for radioactivity.

Figure 2 shows that explanted stubs incorporated ^{45}Ca during regeneration *in vitro* in a manner similar to that of stubs *in vivo*. These explants also showed a two-day lag period during which relatively little ^{45}Ca was incorporated. Thereafter, a rapid increase in radioactivity was observed similar to that shown in Figure 1. A two-day lag period before rapid incorporation of ^{45}Ca was also observed in additional experiments carried out for up to five days with stubs incubated only *in vitro*.

To avoid the two-day lag period so that short term experiments could be conducted while calcification was in progress, spines were fractured on three urchins and allowed to regenerate *in vivo* for four days in plain sea water. One half of the number of stubs was then explanted from each urchin and incubated *in vitro* simultaneously with the remainder *in vivo* in 300 to 350 milliliters of sea water containing 1.0 μc/ml of ^{45}Ca. Ten stubs were sampled from both groups from each urchin at intervals up to 24 hours and assayed for radioactivity.

Figure 3 shows that ^{45}Ca incorporation by calcifying stubs over 24 hours was directly proportional to the length of incubation with no statistically significant difference between stubs incubated *in vivo* and those *in vitro*. Under conditions of the experiment, ^{45}Ca activity was detected in both groups after incubation for as little as one hour.

The results of these experiments indicate that, after regeneration for four days *in vivo* following fracture, explanted stubs could be used alone in further studies on calcification with ^{45}Ca as a tracer. The use of explants is advantageous since removal of ^{45}Ca from the medium due to uptake by the urchin is avoided. In addition, each urchin provides numerous regenerating stubs which can be presumed to be similar genetically and physiologically. Thus, it should be possible to conduct controlled experiments *in vitro* to evaluate quantitatively the effects of various parameters on calcification rates. In the present investigation, two parameters, (1) temperature, and (2) Diamox (acetazolamide), an inhibitor of the enzyme, carbonic anhydrase, were selected for study.

Effect of temperature on calcification rates in vitro

To determine the influence of temperature on calcification rates of regenerating spines *in vitro*, five dishes containing 200 milliliters of sea water and 1.5 μc/ml of ^{45}Ca were allowed to equilibrate for 24 hours at temperatures of 4.7°, 9.7°, 15°, 20°, and 26° C. Fifty stubs were explanted from each of four urchins after regeneration for four days *in vivo* in plain sea water following fracture. Ten stubs from each group of fifty were placed in each of the five dishes giving forty stubs per dish. The four groups of ten stubs in each dish were isolated from one another by pieces of glass rod of $\frac{1}{4}$ inch diameter placed on the bottom of each dish. Temperatures varied by 1° C or less during the experiment. After incubation for 24 hours, all stubs from each animal were assayed for radioactivity by liquid scintillation.

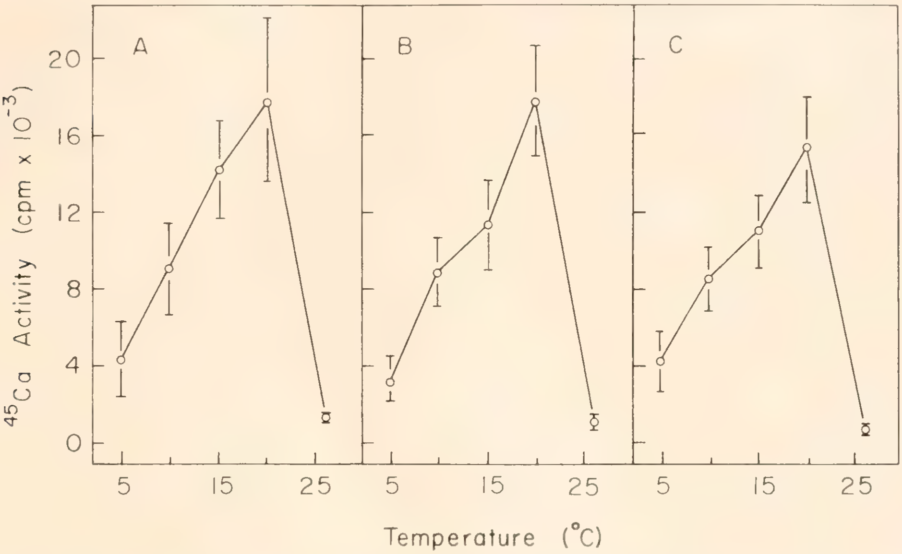

FIGURE 4. Incorporation of ^{45}Ca by spines of three specimens of *S. purpuratus* (A–C) incubated *in vitro* for 24 hours at various temperatures after regeneration for four days *in vivo* in plain sea water following fracture. Each point represents the mean value of 10 spines, with vertical bars indicating ± S.D. Data for a 4th urchin are similar to those shown, except that only a slight increase in ^{45}Ca incorporation occurred between 15° and 20° C.

Figure 4 shows that incorporation of ^{45}Ca by explants was directly proportional to temperature between 4.7° and 20° C, at which a maximum occurred. At 26° C, little incorporation of the label took place, and tissue on all stubs appeared to be partially decomposed at the end of the experiment. Mean values for the temperature coefficient (Q_{10}), and energy of activation (E) calculated over the intervals 4.7° to 9.7°, 9.7° to 15°, and 15° to 20° C, are 5.50, 2.08, 1.73; and 26,052, 11,491, and 8662 calories per mole, respectively, with overall means between 4.7° and 20° C, of 2.72, and 15,504 calories per mole, respectively, for the four urchins studied. Activity of ^{45}Ca measured in the second millimeter of the shaft of those stubs assayed was considered negligible.

Effect of Diamox on calcification rates in vitro

In living systems, carbonate ion ($CO_3^{=}$) can be obtained via bicarbonate ion (HCO_3^-), which is formed by the hydration or hydroxylation of CO_2 (see Wilbur, 1964), although it is not known with certainty which of these two mechanisms is operative at physiological pH (Maren, 1967). The enzyme, carbonic anhydrase, has been shown to catalyze the conversion of CO_2 to HCO_3^- (Meldrum and Roughton, 1933). If the rate of deposition of $CaCO_3$ is dependent upon the enzyme-catalyzed conversion of CO_2 to HCO_3^-, then the application of an appropriate inhibitor of carbonic anhydrase should depress the rate of calcification.

To test this hypothesis in calcifying sea urchin spines *in vitro,* a specific inhibitor of carbonic anhydrase, 2-acetylamino-1,3,4, thiadiazole-5-sulfonamide (Miller, Dessert, and Roblin, 1950), or acetazolamide, was obtained as the sodium salt, Diamox, from the Lederle Laboratories of the American Cyanamid Company, Pearl River, New York. A sea water solution of 4×10^{-3} *M* Diamox was adjusted to pH 8.0 and added to plain sea water in four plastic dishes giving final concentrations of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} *M*. A fifth dish without added inhibitor served as the control. The final volume in all dishes was 250 milliliters, with 1.0 μc/ml of ^{45}Ca. Fifty stubs were explanted from each of three urchins after regeneration for four days *in vivo* in plain sea water following fracture. Ten stubs from each group of fifty were placed in each of the five dishes giving thirty stubs per dish. The three groups of ten stubs in each dish were isolated from one another by pieces of glass rod as described earlier. Incubations were carried out at 15° C for 24 hours. All stubs from each animal were then assayed for radioactivity by liquid scintillation.

TABLE I

The effect of various concentrations of a carbonic anhydrase inhibitor, Diamox (acetazolamide), on the incorporation of calcium-45 by spines of S. purpuratus *incubated* in vitro *for 24 hours after regeneration for four days* in vivo *in plain sea water following fracture. Shown are mean values in counts per minute ± S.D., for the number of spines in brackets. Probability values equal 0.01 or less (see text for statistical procedures).*

Animal	Control	Counts per minute			
		10^{-6} *M* Diamox	10^{-5} *M* Diamox	10^{-4} *M* Diamox	10^{-3} *M* Diamox
1	9596 ± 2404 (10)	3316 ± 968 (10)	4364 ± 2372 (10)	5080 ± 1604 (10)	4908 ± 2052 (10)
2	9000 ± 3102 (9)	3928 ± 1008 (10)	3544 ± 1392 (10)	5060 ± 1472 (10)	4280 ± 1656 (7)
3	9952 ± 1036 (10)	3764 ± 920 (10)	3784 ± 880 (10)	3968 ± 540 (10)	5112 ± 1344 (10)
Mean % inhibition ± S.E.		61 ± 5	58 ± 4	50 ± 9	53 ± 8

Table I shows that there was a statistically significant reduction in ^{45}Ca incorporation by explants treated with the inhibitor compared to the untreated controls. Probability values of 0.01 or less were obtained using the "t" test (Simpson, Roe, and Lewontin, 1960, page 176). The data show a fairly consistent effect of Diamox over a wide range of concentrations with a maximum mean inhibition of 61% at a concentration of 10^{-6} *M*.

DISCUSSION

The results of kinetic studies reported here show that regenerating spines of the sea urchn, *S. purpuratus,* incorporate ^{45}Ca *in vitro* in a manner similar to that of spines from the same animal incubated simultaneously *in vivo.* The data demonstrate a lag period of about two days following experimental fracture before calcification is initiated. The lag period cannot be attributed to the time required for uptake of ^{45}Ca by the urchin, since a similar lag was also shown to occur during regeneration of fractured spines *in vitro.* The results of histological and histochemical studies of fractured spines during regeneration *in vivo* and *in vitro* indicate that the lag more likely reflects the time required for wound healing and reorganization of tissue at the site of fracture (Heatfield, unpublished). Following the lag period, a rapid incorporation of ^{45}Ca takes place *in vivo* and *in vitro.* The incorporation of ^{45}Ca following the lag period parallels the appearance and growth of new mineral in the form of "micro-spines" on the fractured surface of the spine shaft as observed with the light and scanning electron microscopes (Heatfield, 1969). In calcifying stubs which were prepared by allowing fractured spines to regenerate for four days *in vivo* in plain sea water, incorporation of ^{45}Ca *in vitro* was shown to be equivalent to that *in vivo* during incubation for 24 hours. The nearly linear rate of incorporation of the label during this period indicates that equilibrium between the tissues of regenerating spines and the incubation medium takes place rapidly (less than one hour at the spine tip).

Using explanted, fractured spines as a calcifying system after regeneration for four days *in vivo*, temperature was shown to have a marked effect on calcification rates *in vitro.* Incorporation of ^{45}Ca was directly proportional to temperature between 4.7° and 20° C, at which a maximum occurred. Little incorporation of the label took place at 26° C, which appears to be a lethal temperature. The temperature of the ocean in the vicinity of the collecting site ranges from about 13° to 20° C (Boolootian, 1961). This range is within that in which incorporation of ^{45}Ca was found to occur in the present work and indicates that temperature is not a limiting environmental factor in the regeneration process in spines of *S. purpuratus.* The temperature coefficient (Q_{10}) and the energy of activation (E) varied inversely with increasing temperature betwen 4.7° and 20° C. Valus of E obtained in the present study fall within the general range (5000 to 25,000 calories per mole, and higher) listed by Sizer (1943) for a large number of enzyme-catalyzed reactions, and by Crozier (1924) for a variety of physiological phenomena. In the present work, the variation of E (μ of Crozier) with temperature suggests that several rate-limiting reactions may be involved in the calcification process with the one in effect at any particular moment dependent upon the ambient temperature (see Crozier, 1924).

Few quantitative studies have been carried out on the effect of temperature on mineral deposition in other calcifying systems. Malone and Dodd (1967) found that incorporation of ^{45}Ca into whole shells of the bivalve mollusc, *Mytilus edulis,* varied directly with temperature over the range, 5° to 23.5° C. Incorporation due to exchange was not estimated and it was concluded that activity of ^{45}Ca in shells incubated at the lower temperatures might not be due to skeletogenesis. In the present study, incorporation of ^{45}Ca at 4.7° C was about $\frac{1}{4}$ of the maximum observed at 20° C, indicating that significant deposition of mineral takes place in regenerating spines of *S. purpuratus* even at relatively low temperatures. Porcella, Rixford, and Slater (1969) found that uptake of ^{45}Ca by the fresh water crustacean, *Daphnia magna*, was temperature dependent, and obtained mean values for E of 15,000 calories per mole between temperatures of 10° and 25° C. In the study reported here, a similar mean value of E of 15,504 calories per mole was obtained between 4.7° and 20° C. In the reef coral, *Pocillopora damicornis*, Clausen (unpublished, cited in Lenhoff, Muscatine, and Davis, 1968) measured the effect of temperature on the incorporation of ^{45}Ca, and found an exponential increase in radioactivity between 12° and 25° C, with a Q_{10} of 12.7 or 6.7 depending on the length of incubation. Incorporation at 12° was about 1/13 that at 25° C. Above 25° C, a decline in calcification rate was observed. Values for E of 43,000 and 33,000 calories per mole were obtained by Clausen depending on the length of incubation. These values are about twice as high as those obtained in the present study and by Porcella *et al.* (1969), and indicate that the rate of calcification in this species of coral is very sensitive to changes in temperature.

The enzyme, carbonic anhydrase, is generally believed to be important in the biological deposition of calcium carbonate as inferred from the results of experiments on the effect of carbonic anhydrase inhibitors on the rate of mineral deposition. The data reported here show a statistically significant reduction in the incorporation of ^{45}Ca by regenerating spines of *S. purpuratus* incubated *in vitro* in the presence of a carbonic anhydrase inhibitor, Diamox (acetazolamide). Inhibition of 50% to 61% was obtained over a concentration range of 10^{-3} to 10^{-6} *M*. From these results it is inferred that carbonic anhydrase plays a role in calcification of regenerating spines of *S. purpuratus* though no attempt has yet been made to determine the presence of this enzyme in spine tissues. Inhibition of calcification has been observed in other organisms in which carbonic anhydrase activity was detected. Wilbur and Jodrey (1955) obtained up to 48% inhibition of ^{45}Ca incorporation by the oyster, *Crassostrea virginica*, in the presence of 4×10^{-5} *M* Diamox. Costlow (1959) observed an inhibition of shell growth in the barnacle, *Balanus improvisus*, after treatment with Diamox at concentrations ranging from 3.7×10^{-4} to 2.99×10^{-5} *M*. In four species of reef coral, Goreau (1959) found an inhibition of ^{45}Ca incorporation of 51% to 80% in experiments conducted in the light with Diamox at a concentration of 10^{-3} *M*. Isenberg, Lavine, and Weissfellner (1963) obtained essentially complete inhibition of mineralization in the coccolithophorid, *Hymenomonas* sp. after exposure to 10^{-3} *M* Diamox, though the demonstration of carbonic anhydrase was not attempted in this organism. After injection of Diamox into the hen, *Gallus domesticus*, Bernstein, Nevalainen, Schraer, and Schraer (1968) measured a reduction in egg-shell weight of approximately 59%.

The results of these studies with Diamox, with the possible exception of Costlow (1959) and Isenberg *et al.* (1963), indicate that carbonic anhydrase is generally not indispensable to skeletogenesis, since deposition of mineral occurs, though at a reduced rate. Concentrations of Diamox which have a marked effect on calcification rates do not appear to be toxic (see Wilbur and Jodrey, 1955; Costlow, 1959; Goreau, 1959; Freeman, 1960; Isenberg *et al.* (1963); and Maren, 1967). In the presence of another inhibitor, 2-benzothiazolesulfonamide, Stolkowski (1948) observed an inhibition of growth in the larval skeleton of the sea urchins, *Paracentrotus lividus* and *Arbacia aequituberculata*. However, complete inhibition of mineral deposition was obtained with this drug in *C. virginica* by Wilbur and Jodrey (1955), and in the fresh water snail, *Physa heterostropha*, by Freeman (1960), which led these workers to conclude that the action of 2-benzothiazolesulfonamide was not limited to carbonic anhydrase.

In calcifying systems where deposition of mineral proceeds at a slow rate and the enzyme-catalyzed hydration or hydroxylation of CO_2 is not rate-limiting, then the application of inhibitors of carbonic anhydrase may not show an effect. Such a result was obtained by Freeman (1960) in studies of shell growth of *P. heterostropha* grown at different rates in the presence of Diamox.

It is a pleasure to acknowledge the enthusiasm, support, and guidance shown throughout this work by Dr. Leonard Muscatine of the Department of Zoology, University of California, Los Angeles. I would also like to thank Dr. Karl M. Wilbur of the Department of Zoology, Duke University, for critically reading the manuscript.

SUMMARY

1. Calcification during regeneration of experimentally fractured spines of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson), was studied quantitatively under different conditions with calcium-45 as a tracer.

2. Fractured spines rapidly incorporated ^{45}Ca *in vivo* or *in vitro* after a lag period of about two days. The lag period is attributed to wound healing and reorganization of tissue at the site of fracture.

3. Additional experiments were conducted while calcification was in progress by allowing fractured spines to regenerate for four days *in vivo* followed by incubation in ^{45}Ca *in vivo* or *in vitro* up to 24 hours. In these experiments incorporation of the label was nearly linear with time and no significant difference was observed in the rate of uptake of ^{45}Ca between regenerating spines incubated *in vivo* and those from the same urchin incubated simultaneously *in vitro*.

4. Incorporation of ^{45}Ca *in vitro* was directly proportional to temperature between 4.7° and 20° C, at which a maximum occurred. A temperature of 26° C appeared to be lethal and little incorporation of ^{45}Ca took place. Values of Q_{10} and the energy of activation varied inversely with temperature, with overall means of 2.72 and 15,504 calories per mole, respectively, between 4.7° and 20° C.

5. Diamox (acetazolamide) at concentrations from 10^{-3} to 10^{-6} *M*, inhibited incorporation of ^{45}Ca *in vitro* by 50% to 61%. It is inferred from these results that carbonic anhydrase is involved in calcification of regenerating spines of *S. purpuratus*.

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A COMPARATIVE STUDY OF THE CEMENT GLANDS IN SOME BALANID BARNACLES (CIRRIPEDIA, BALANIDAE)[1]

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The structure of the cement glands in Cirripedia was only briefly mentioned by Darwin (1854), Krohn (1859), Koehler (1889), Gruvel (1905a, b) and a few others. Recently, beginning with Thomas (1944), several authors studied various types of epidermal glands in this group of animals (Boucquet-Vedrine and Ovechko, 1960; Utinomi, 1960; Walley, 1967), no one, however, dealt with the specialized glands responsible for the secretion of cement. A detailed histological study by Lacombe (1966) on adult specimens of *Balanus tintinnabulum* called attention to the ectodermal origin of the cement glands. Subsequent histochemical investigations (Lacombe, 1967a) suggested that the intra- and extracellular secretion of the cement glands may be a type of acid mucopolysaccharide. Arvy and Lacombe (1968) and Arvy, Lacombe and Shimony (1968) utilizing histoenzymological techniques showed that the secretion spread in the cell and the extruded secretion within the canal system give a positive reaction for alkaline phosphatase, but the rest of the cement gland cell remains negative. A comparative study of the cement gland in *B. tintinnabulum* and *Lepas anatifera* (Lacombe and Liguori, 1969) suggested that the structure of the cement glands may be less complex in the primitive barnacles, such as the *Lepalidae*.

METHODS

The ovaria with the cement glands of adult specimens of *B. nubilis* and *B. psittacus* were dissected for immediate fixation; in the case of *B. eburneus, B. amphitrite* and *B. balanoides* the entire animal was fixed.

The following fixatives were employed: Flemming, Bouin, Susa, Carnoy, Helly, and Altmann's fluid, as well as 10% formaldehyde. The material was embedded in paraffin and sections were cut at 5, 7 and 10 μ.

The sections were stained with Delafield's hematoxylin and Chromotrop 2R, Ehrlich's hematoxylin and Orange G. G., Weigert's hematoxylin and Alcian blue at pH 2.3, Mallory's Azan method with Congo red, Trypan blue with Chromotrop 2R, Aniline blue and Nuclear fast red with Naphthol green. For fine details Heidenhain's iron alum hematoxylin was used, following fixation in Flemming's fluid.

OBSERVATIONS

Typically, as in *B. tintinnabulum* (Lacombe, 1966), the secretory cells which elaborate the cement occur in groups (*B. psittacus, B. eburneus* and *B.*

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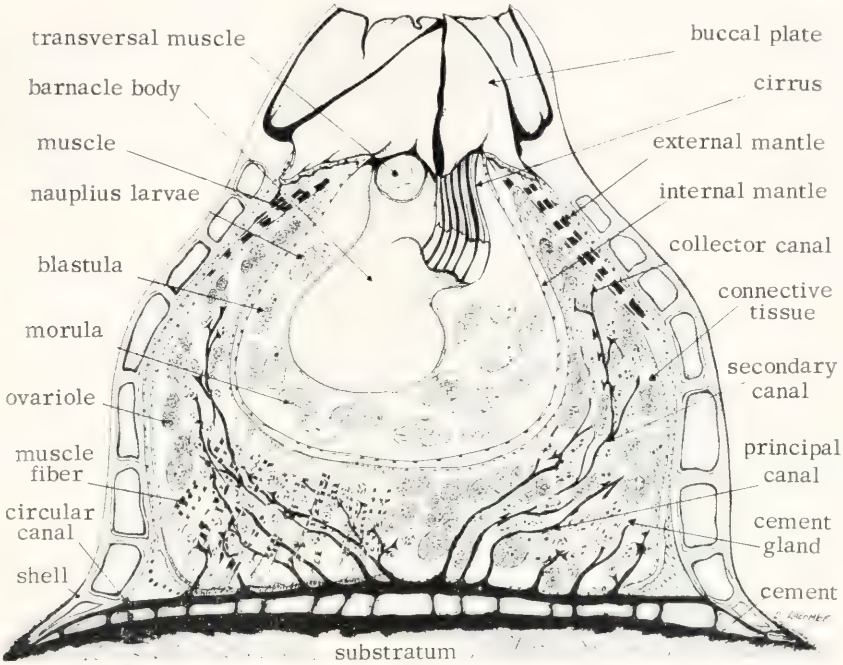
[1] Translated from the Portuguese by Dr. Sophie Jakowska, Universidad Autonoma de Santo Domingo, Dominican Republic.

amphrite) or in rosettes (*B. nubilis*) intermingled with the ovarian follicles. In *B. balanoides*, on the other hand, they are scattered individually among the connective tissues in the basal portion of the animal. The number of cement gland cells appears relatively smaller in *B. balanoides* than in the other species examined.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barnacle body
muscle
nauplius larvae
blastula
morula
ovariole
muscle fiber
circular canal
shell
buccal plate
cirrus
external mantle
internal mantle
collector canal
connective tissue
secondary canal
principal canal
cement gland
cement
substratum

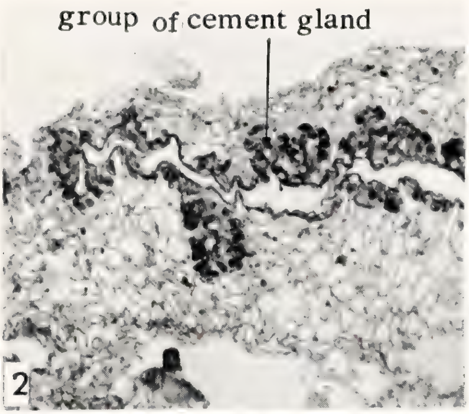
FIGURE 1. Schematic drawing of a longitudinal section in *Balanus* sp., showing the internal anatomy and cement glands with canal system.

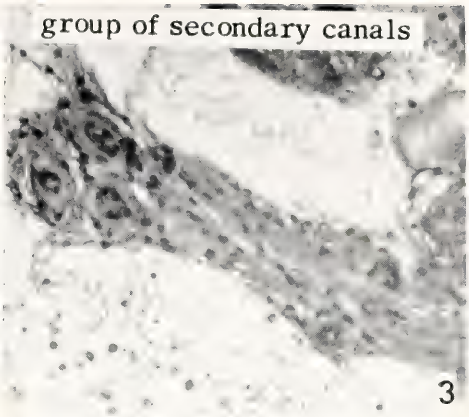
Figure 1 is a schematic drawing of the anatomy of the typical balanid barnacle in longitudinal section. The structures include the cement glands among the ovarian follicles, the canal system, through which the cement secretion is conveyed to the basal portion of the animal, and the flat epithelium of the mantle, shown in lateral view.

A. Secretory elements

In *B. nubilis* the cement glands are found in the connective tissue near the ovarian region. In the external zone of the mantle the glands appear to be in the early stages of development (Fig. 2), and consist of small cells differentiating in the walls of the principal and secondary canals (Fig. 3). The young cement glands form groups of 15 to 30 small cells which have dense homogeneous cytoplasm and a central nucleus rich in chromatin.

During their development the gland cells become embedded deeper in the interior of the mantle-chamber and located in the connective tissue near the


group of cement gland
2


group of secondary canals
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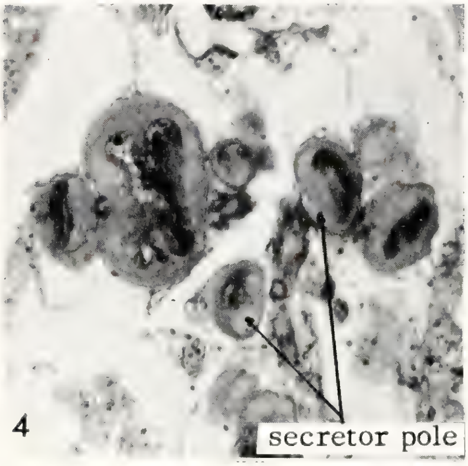
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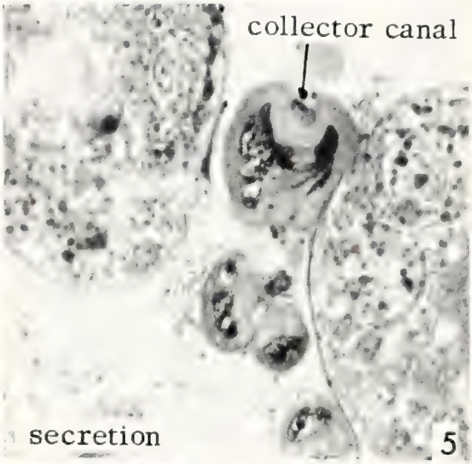
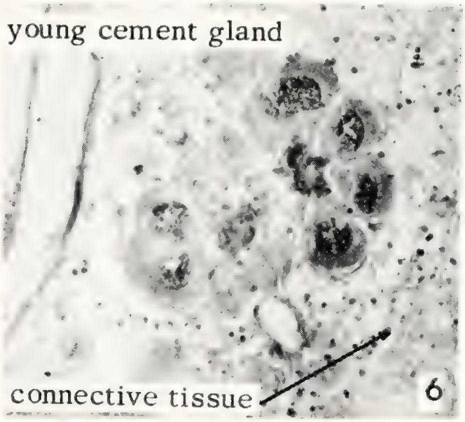

collector canal
secretion
5


young cement gland
connective tissue
6

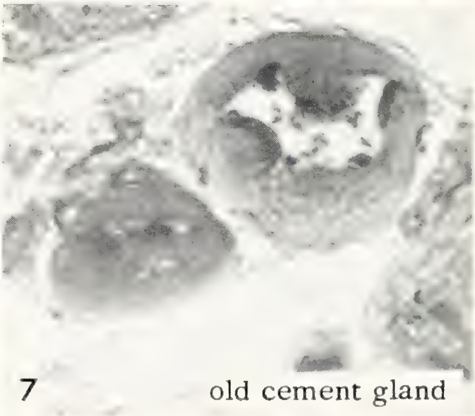

7
old cement gland

FIGURE 2. *Balanus nubilis.* Longitudinal section through secondary canal with young cement glands in development, Mallory-Azan.

FIGURE 3. Transverse section through principal canals near the hilum region, Mallory-Azan.

FIGURES 4 to 7. Transverse section through the cement glands in different stages; nuclear fast red and naphthol green.

ovarian follicles (Figs. 4 to 7). At this stage the glands begin to elaborate the cement secretion (Fig. 4). The secretion originates in one zone of the cell, the secretory pole, and accumulates in another zone, the storage pole; the latter connects with the collector canal (Fig. 5); that means, from the point of view of genesis of this type of cells, that the apical part of the gland cell stores the secretion and the basal part produces it. Occasionally the cells of the canal walls develop clusters of secretory cells which appear as partial or complete rosettes in histological sections (Fig. 6).

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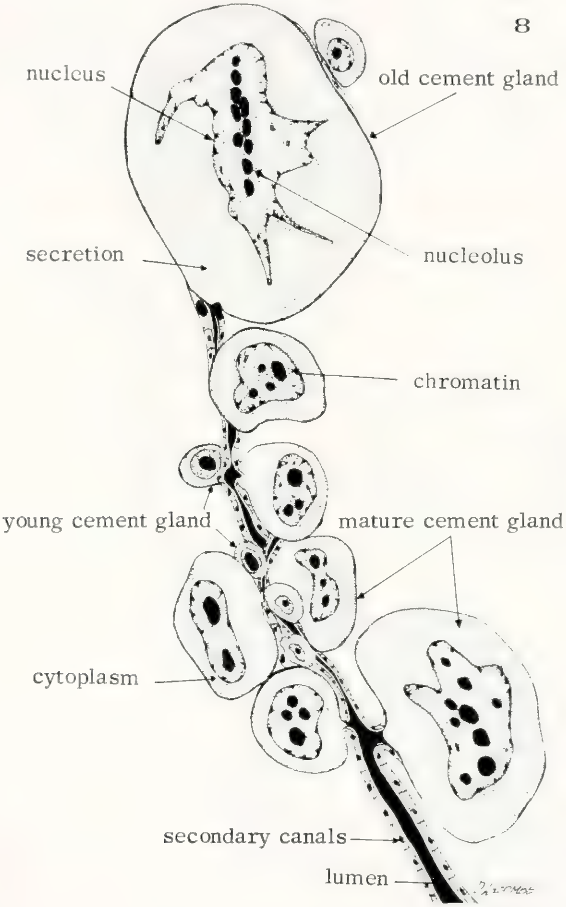
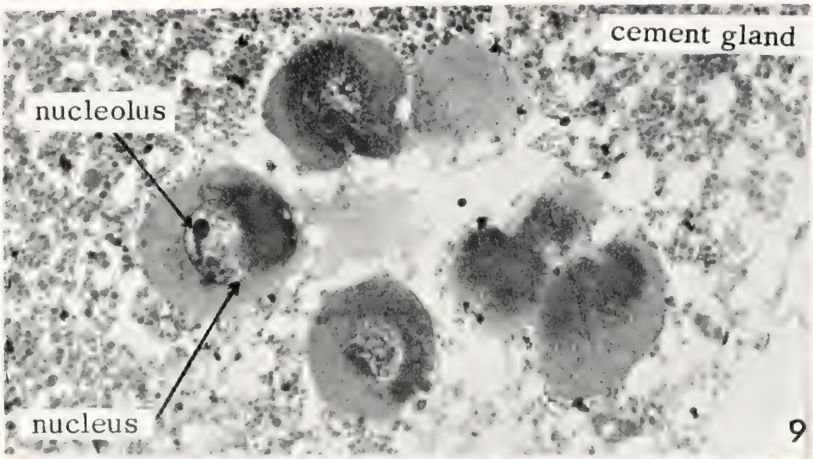
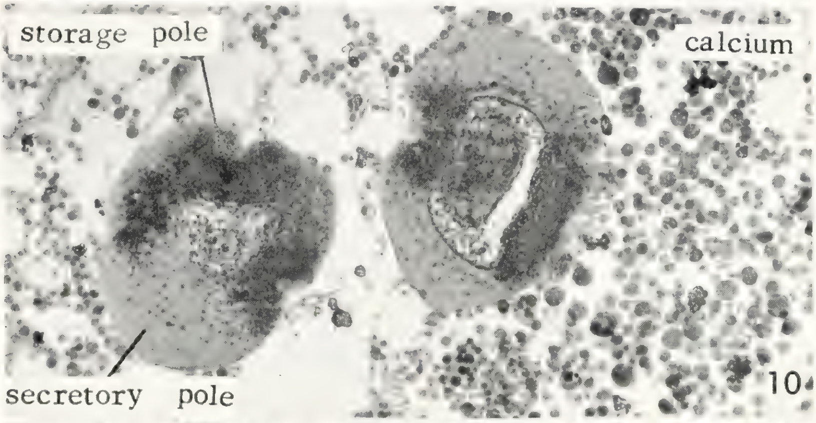

nucleus
old cement gland
secretion
nucleolus
chromatin
young cement gland
mature cement gland
cytoplasm
secondary canals
lumen

FIGURE 8. Cement glands in *Balanus psittacus* showing different stages of development.

Within such clusters the cement secretion is clearly visible. Young cells forming the clusters have regular, centrally located nuclei, each with two to four nucleoli. In mature cells, the nuclei are polymorphic, with many nucleoli in varied positions. Older cement gland cells have large and more irregular nuclei, and an even larger number of nucleoli; the nuclei are very poor in chromatin indicating an increase of nucleoplasm.


cement gland
nucleolus
nucleus
9


storage pole
calcium
secretory pole
10

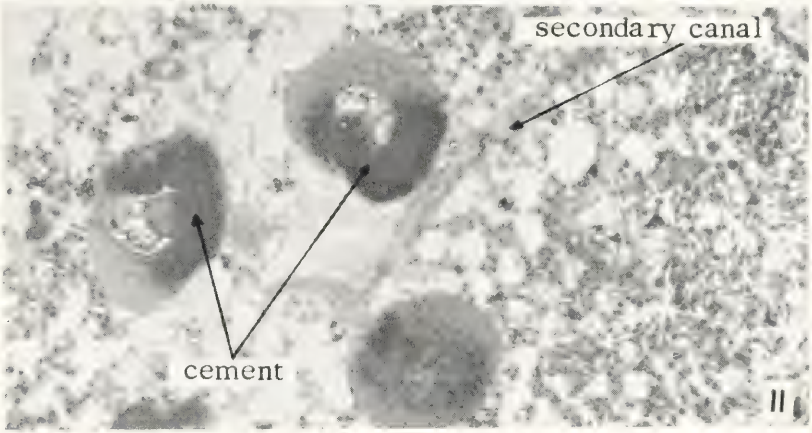

secondary canal
cement
11

FIGURE 9. *Balanus eburneus.* Intercellular cement secretion; nuclear fast red and Azan method.

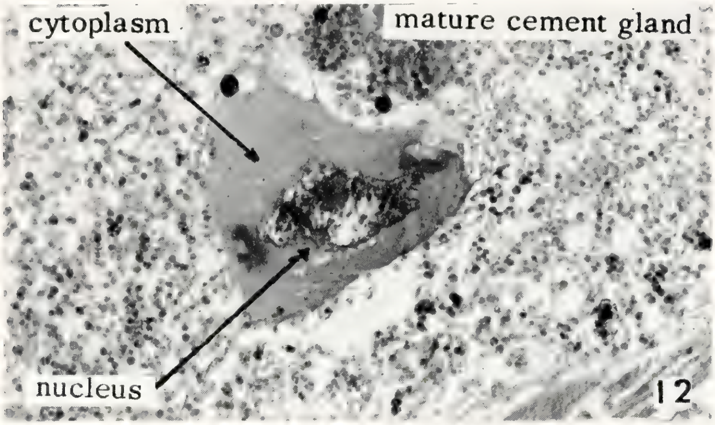
FIGURE 10. Same gland in higher magnification.

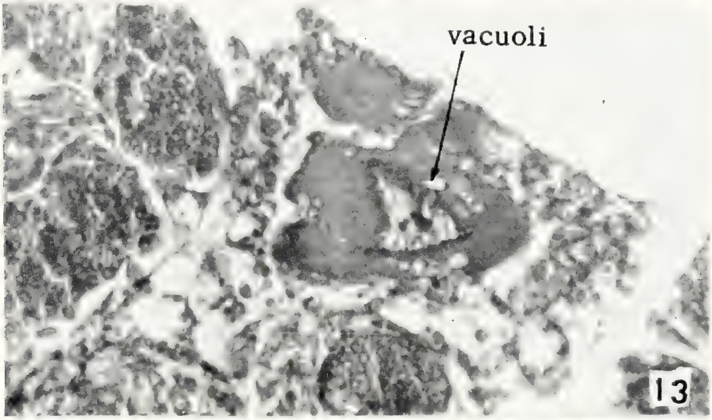
FIGURE 11. Longitudinal section through the secondary and collector canals.

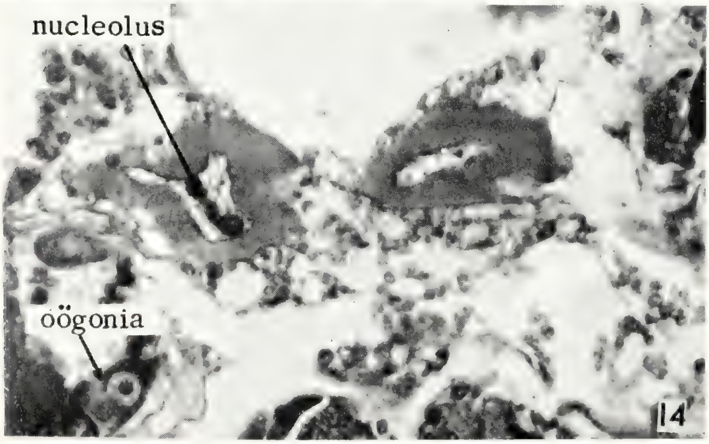
In *B. psittacus* the cement glands are much easier to locate than in *B. nubilis.* In both species the epithelium of the mantle occupies a large part of the animal's shell. In the walls of the principal and secondary canals the gland cells grow outward from the lumen. All stages of cell development and maturation may be easily seen (Fig. 8). The cytoplasm of the gland cells exhibits very small vacuoles which increase in size as the cells mature. These vacuoles, full of cement, are distributed throughout the cytoplasm. When stained with the Mallory's Azan method, or with nuclear fast red stain and Alcian blue, the cement gland cells take on a uniform coloration. In *B. psittacus,* an accumulation of the cement at the storage pole, such as in *B. nubilis,* was not observed. The nuclei of the gland cells are large and more polymorphic, with 10–14 nucleoli irregularly distributed through the nucleoplasm. The nuclear chromatin is poor and dispersed. Each group of cement glands is composed of 8 to 14 secretory units or cells in different stages of development.

The gland in *B. eburneus* consists of a few, well-defined cells (Fig. 9). The young gland cells are located very close to the basal plate or the point at which the gland cells begin to differentiate from the canal walls. The mature gland cells are located in the connective tissue among the ovarian follicles. In this species the gland cells do not change in shape and form during the development. The cytoplasm is dense, homogeneous and devoid of vacuoles. The nuclei are constant in form and exhibit very little polymorphism; chromatin is mostly concentrated around the nucleoli, which number two to four. The cement secretion is visible as granular particles scattered in the cytoplasm at the secretory pole of the cell (Fig. 10). At the storage pole the secretion granules are very dense and they leave the cell through a fine membrane, passing into the lumen of the collecting duct (Fig. 11). A similar condition was observed in the cement gland of *B. tintinnabulum* (Lacombe, 1966). At the storage pole of each gland cell of *B. eburneus,* it is seen with the light microscope that the cytoplasm projects into the nucleus as shown in Fig. 10; this condition results in an increase of surface of contact between the secretion and the nuclear elements. In *B. tintinnabulum,* the electron microscope revealed that the endoplasmic reticulum has a different arrangement in this region of contact (Lacombe, 1967b), and suggested a relationship between the nuclear elements and the cement secretion.

The cement glands of *B. balanoides* differ distinctly from those of all the other species described in this paper. In this species the glands (Fig. 12) are located in the basal portion of the animal near the region where the shell increases in size. They never appear as a distinct group of cells or as rosette shaped clusters. The glands consist of single isolated cells and the mature elements are particularly conspicuous in the connective tissue. Young cement glands are rarely seen in adult *B. balanoides,* perhaps due to the fact that the growth of the glands closely parallels that of the animal. Another distinctive feature of these gland cells is the abundance of cytoplasmic vacuoles containing cement secretion (Fig. 13), which gradually increase in size. When many vacuoles containing secretion accumulate, they move toward the cell membrane nearest to the collector canal. As it was observed in *Lepas anatifera* (Lacombe and Liguori, 1969), *B. balanoides* exhibits more than one way of discharging cement secretion into the lumen of the collector canals, and this corresponds to the primitive condition. Figure 12 clearly shows two typical storage and discharge points in a cement gland cell.


cytoplasm
mature cement gland
nucleus
12




nucleolus
oögonia
14

FIGURES 12 and 13. *Balanus balanoides.* Cement glands showing the vacuoles and the paths of cement extrusion; Erlich's hematoxylin and chromotrope 2R.

FIGURE 14. Cement glands, with typical nucleoli in the polymorphic nuclei; Heidenhain's Iron hematoxylin.

The cement glands of *B. balanoides* exhibit considerable nuclear polymorphism such as seen in *B. eburneus*. The number of nucleoli varies from four to fourteen and they are clearly defined (Fig. 14). The schematic drawing (Fig. 15) shows different parts of this type of cell.

In *B. amphitrite,* the cement glands closely resemble those of *B. eburneus* in size, localization and form. The glands of *B. amphitrite* are located among the ovarian follicles (Fig. 16) and most frequently near the white muscles. The glands are composed of groups of 6 to 10 cells and are seldom represented by single isolated cells (Fig. 17). During all stages of development the gland cells are regular in form; their cytoplasm is dense and contains small vacuoles near the cell membrane. Usually different stages of developing cells are found growing among the old gland cells (Fig. 18). This condition differs from that observed in *B. balanoides.* The occurrence of differences of cell development simplifies the observation of the cytology of the various cell phases. In young gland cells, the nuclei are round, poor in chromatin and have centrally placed nucleoli. In mature glands, however, the nuclei tend to become polymorphic. The cement secretion of *B. amphitrite* is similar to that of *B. eburneus,* and both species show very distinct storage and secretory poles in the mature gland cells.

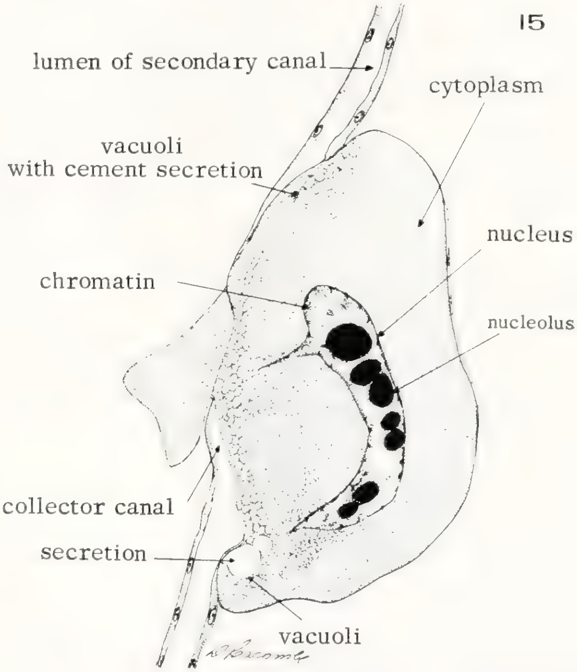
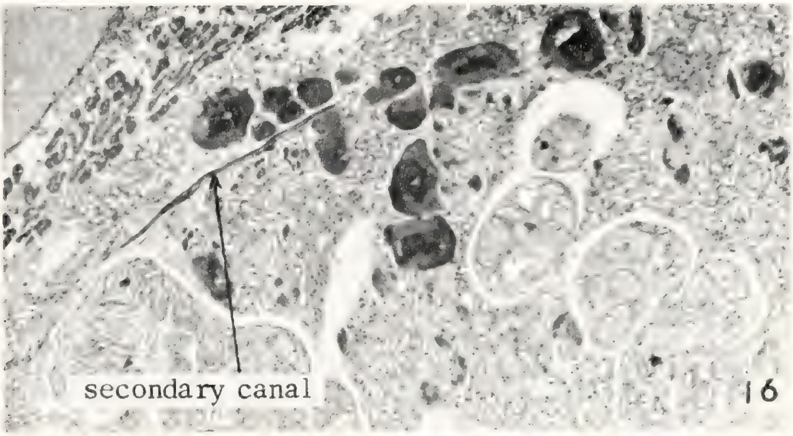
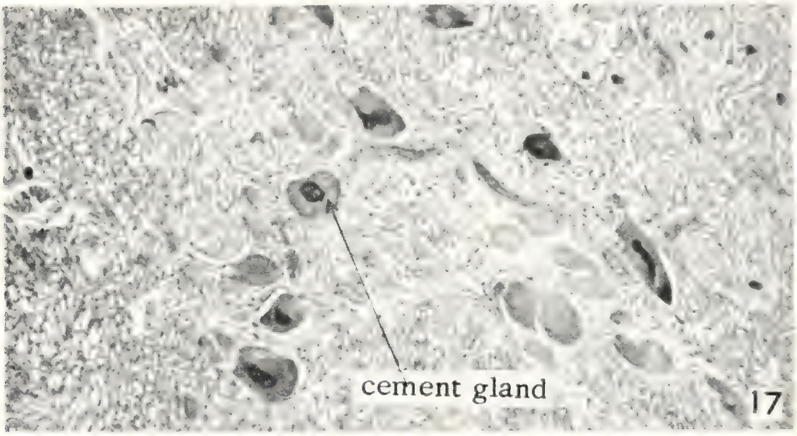
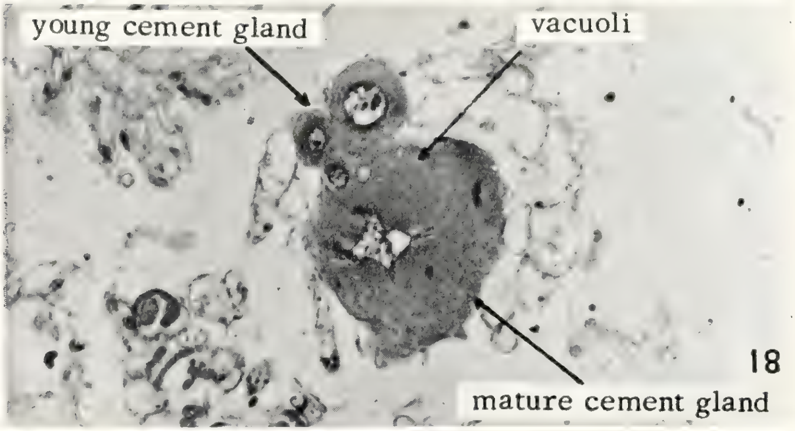


FIGURE 15. Cement gland of *Balanus balanoides,* showing vacuoles rich in cement secretion, nucleoli of different sizes in the polymorphic nuclei.


secondary canal
16


cement gland
17


young cement gland
vacuoli
18
mature cement gland

Figures 16 and 17. *Balanus amphitrite.* Groups of cement gland cells with secondary canals, Delafield's hematoxylin and chromatrope 2R.

Figure 18. Different stages of development of cement glands; Erlich's hematoxylin with Congo red and orange G.G.

B. Accessory canal system

The accessory canal system, which distributes the cement, was previously studied in *B. tintinnabulum* (Lacombe, 1966). It originates by progressive invagination of the hypodermal cells of the exterior mantle wall after the cypris larva becomes attached. Thus begins the formation of a complex system consisting of circular, radial and principal canals. The principal canals grow inward and ramify extensively, giving rise to numerous secondary canals. Subsequently, the chitin of the mantle epithelium spreads as a lining over the entire lumen of this canal system. Some cells in the walls of the principal canals or the secondary canals begin to differentiate into the specialized cement glands.

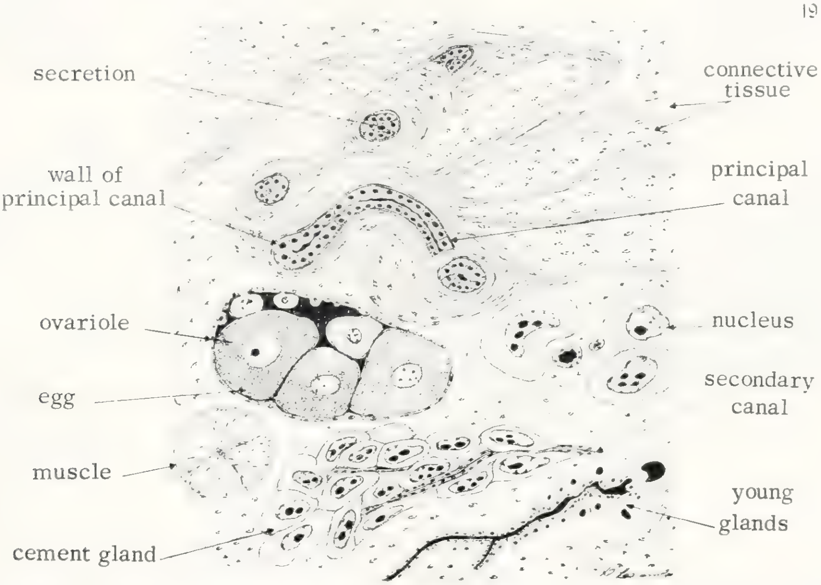
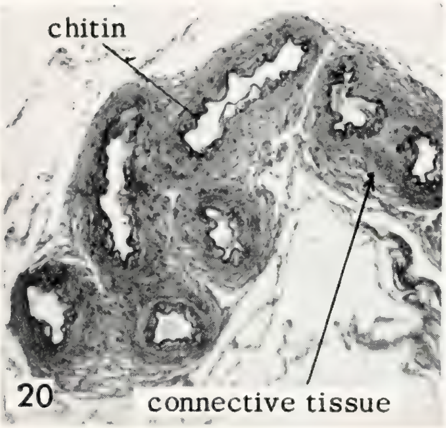
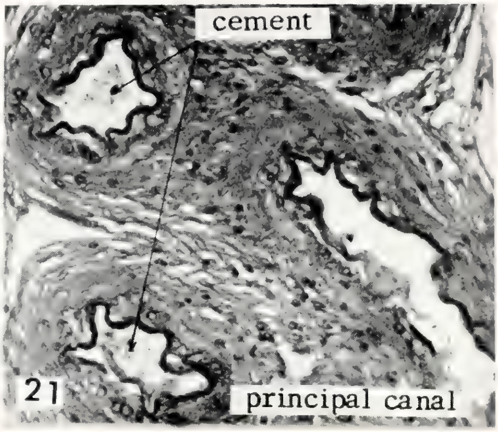


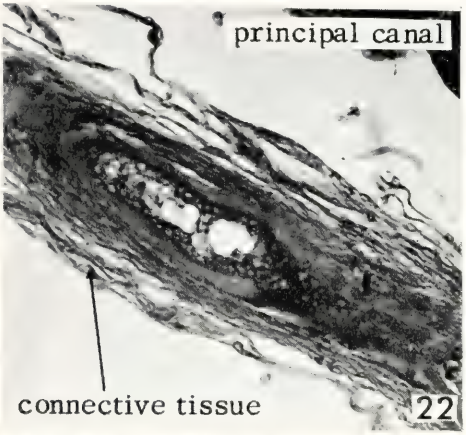
FIGURE 19. Ovarian region in *Balanus nubilis* showing the elastic fibers around the principal and secondary canals, with young, mature and old cement glands and oocytes in development.

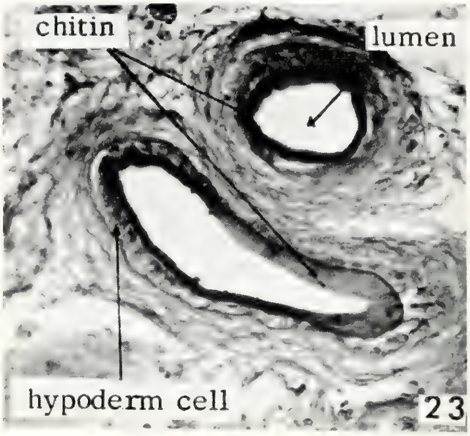
The configuration of the canal system varies to some extent in the five species of balanids studied in this paper, but in general the pattern resembles that of *B. tintinnabulum* or that in the schematic drawing of the internal anatomy of a typical barnacle (Fig. 1). The secretion passes through the cell membrane from the vacuoles or from the storage pole into the lumen of the collecting canals, from where it moves on to the secondary and to the principal canals. The principal canals conduct the secretion to the radial and circular canals, from where it spreads out beneath the basal plate by typical outlets.

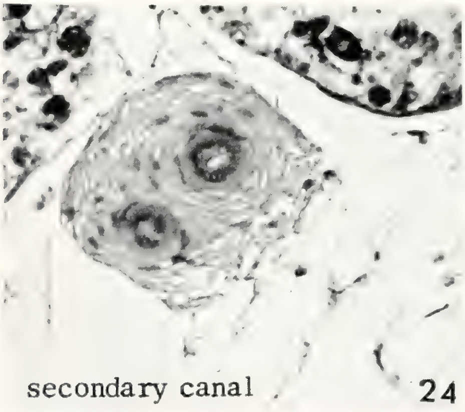
In *B. nubilis* the secondary canals are narrow and many developmental stages of gland cells may be seen in their epithelium (Fig. 19). The principal canals adjoining the ovarian region have a large diameter and their epithelial cells tend to be larger. The connective tissue fibers are concentrated around the principal


chitin
20
connective tissue


cement
21
principal canal


principal canal
connective tissue
22


chitin
lumen
hypoderm cell
23


secondary canal
24

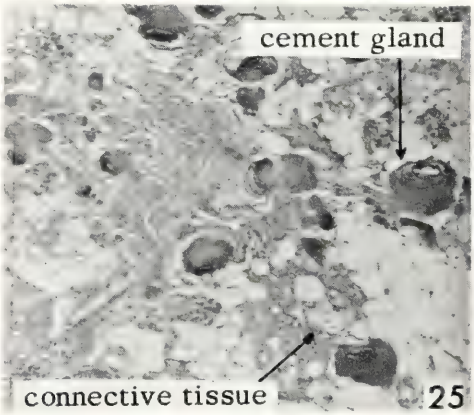

cement gland
connective tissue
25

FIGURE 20. *Balanus psittacus.* Principal canals, showing connective tissue fibers, hypodermal cells and chitin; Mallory's Azan method with Congo red.

FIGURE 21. Same section as in Figure 20 showing the cement secretion in the lumen of the principal canal, same stain.

canals and apparently function as a protective covering (Fig. 19). The chitinous layer within the lumen of the principal canals is very difficult to observe, but it becomes evident and is clearly seen with polarized light. In this species, as well as in *B. psittacus*, a hilum appears in regions where the secondary canals join the principal canals.

The hilum is absent in *B. eburneus, B. amphitrite* and *B. balanoides.* The principal canals are composed of very simple, flat epithelial cells (Fig. 11) with elongated nuclei; these are rich in chromatin, a fact which indicates that these nuclei are out of function, their nucleoplasm was reduced and their chromatin appears now more concentrated. There are no connective tissue fibers around the principal or secondary canals. The arrangement of the canal system is similar to that of *B. tintinnabulum.*

In *B. psittacus* the hilum is greatly accentuated and in this region all the cement gland cells show discrete secretion. The structure of the principal canals, indicated in Figures 20 to 23, resembles that of *B. nubilis* in which the principal canals have a tortuous course (Fig. 20) and the secondary and the principal canals (Fig. 22 and 24) are surrounded by a large number of connective tissue fibers. The latter feature appears to be typical of the larger species of balanids, such as *B. psittacus* and *B. nubilis,* and it is not observed in the others. In *B. psittacus,* the chitinous layer in the principal canals (Fig. 23) is clearly shown by simple staining with Congo red. The hypodermal cells in this species possess dense cytoplasm and a large, basally situated nucleus, poor in chromatin. Figure 25 shows a general histological view of the hilum region when the cement gland cells and canals are filled with secretion. The secretion in this species is clearly visualized along the entire canal system with simple histological stains. This is not as easily observed with such methods in the other balanids studied where only small granulations appear (Fig. 20–21), but histoenzymological methods permit to demonstrate the cement in those species.

C. Extrusion of the cement secretion

In the serial sections of *B. eburneus, B. balanoides* and *B. amphitrite,* stained with Heidenhain's iron alum hematoxylin, the basal muscle of the mantle is easily seen; Gutmann (1960) called attention to these muscles, but did not associate them with the cement glands. These striated muscle fibers extend from the chitinous covering of the external mantle epithelium on the base plate (Fig. 26), to the thin chitin of the inner mantle layer, which covers the body of the barnacle.

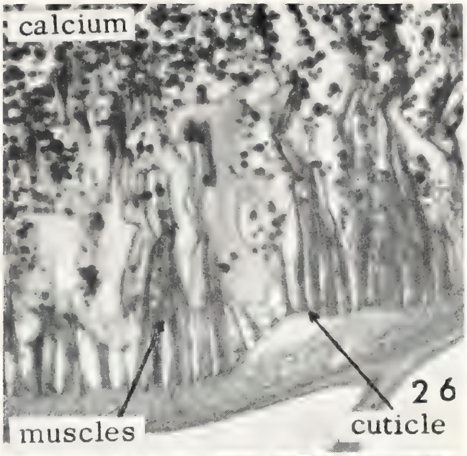
The muscle fibers pass through the connective tissue between the ovarian follicles and the cement glands (Fig. 27). These fibers are very distinct, with long tonofibrils in the base plate (Fig. 26) and very short tonofibrils at the mantle side. The striated muscles are closely associated with the cement glands and ovarian follicles as may be seen in cross section (Figs. 27 and 28). Figure 28

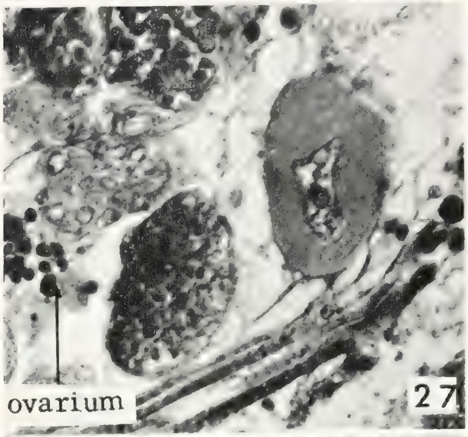
FIGURE 22. Principal canal with cement secretion, same stain.

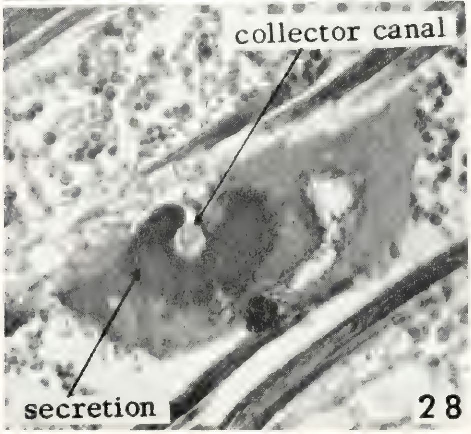
FIGURE 23. Principal canals showing chitin and hypodermal layer and connective tissue transverse section, same stain.

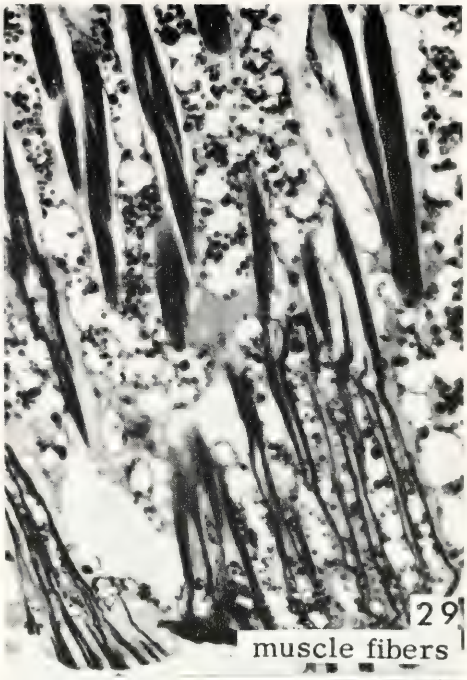
FIGURE 24. Secondary canal near the hilum region, showing distinct connective tissue fibers, transverse section, same stain.

FIGURE 25. General view of the hilum region with cement glands in different stages and the arrangement of the canal system, same stain.


calcium
muscles
cuticle
2 6




collector canal
secretion
2 8


2 9
muscle fibers

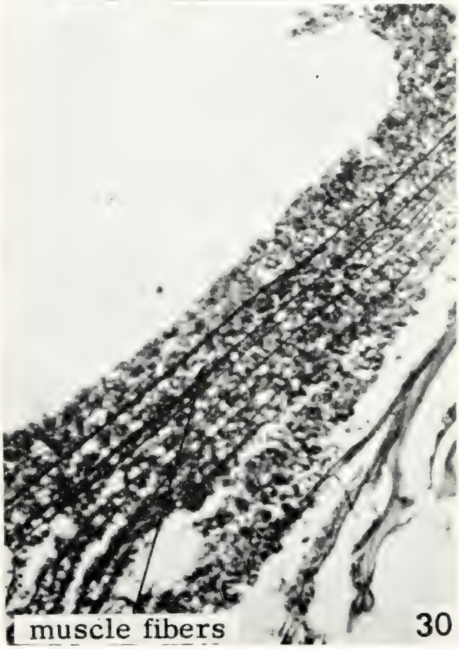

muscle fibers
30

FIGURE 26. Longitudinal section through the base plate of *Balanus eburneus* showing the long tonofibrils and the muscles of the mantle, Heidenhain's iron hematoxylin.

FIGURE 27. The cement gland in *Balanus balanoides* and muscular system of the mantle, same stain.

shows the cement glands of *B. amphitrite*, with the collector canal in cross section next to the storage pole. It might be supposed that this musculature (Figs. 29 and 30) is involved in some way in the movement of the cement secretion from the secondary and principal canals to the basal system. The contraction of these muscle fibers probably compresses the entire ovarian region, which includes the cement glands and the connective tissue, and may cause the cement to move towards the basal canal. Perhaps this contraction helps extrude the cement secretion from the canal system to the substrate in the absence of additional musculature in the walls of the canals. The basic anatomical relationships, seen in Figure 1, suggest the above interpretations.

DISCUSSION

The pattern that seems to emerge from the observations on the Balanids selected for study suggest that the degree of development and differentiation in the cement gland system may be related to the phylogenetic position of the species.

B. balanoides is the species that exhibits a relatively simple pattern: (1) single large secretory cells situated at the base of the animal, away from the ovarian follicles; (2) the secretion scattered throughout the cytoplasm and collected in vacuoles which extend into the collector canal; (3) the absence of elastic fibers around the canals.

B. amphitrite, *B. eburneus* and *B. tintinnabulum* (the last was studied previously by Lacombe, 1966), represent what may be considered as a typical pattern for balanids: 1) large secretory cells, arranged in irregular groups, intermixed with the ovarian follicles; 2) the secretion formed at the secretory pole of the cells and extruded at the opposite pole into the collector conduct without the formation of vacuoles; 3) the absence of elastic fibers in the walls of the canal system.

In contrast to the above species, *B. psittacus* and *B. nubilis* both possess elastic fibers around the canals, and in both, the cement glands are intermixed with the ovarian follicles. In *B. psittacus,* however, the secretory elements consist of irregular groups of small and large cells and in *B. nubilis,* all the cells are small and are arranged in form of rosettes. The secretion is not confined to the vacuoles or to the storage pole, but appears scattered throughout the cytoplasm.

It may be too early to speculate on the significance of these differences observed in the balanids under study. It is generally recognized, however, that *B. balanoides* may represent a primitive form, while the large barnacles, *B. psittacus* and *B. nubilis* may be considered as the more advanced forms of balanids. The morphological differences observed in the cement gland system in these species point to interpretations involving phylogenetic concepts. In addition, one is impressed by the fact that adult specimens of *B. balanoides* exhibit only fully formed cement gland elements, while in the other species one may observe within the same adult specimens the progressive stages of differentiation of these elements.

FIGURE 28. Cement gland and muscle fibers in *Balanus amphitrite,* showing the striated muscles, the storage pole of the gland cell and collector canal in transverse section, same strain.

FIGURE 29. Longitudinal section through the mantle of *B. eburneus,* showing the muscle fibers, same stain.

FIGURE 30. General view of the muscle system of the mantle of *B. eburneus,* same stain.

This may suggest that the maturation of the animal and of the cement gland elements may be synchronized with the molting cycle in the more primitive, but not in the more advanced balanids.

B. eburneus and *B. tintinnabulum* appear to be highly suitable for further demonstration, histochemically and ultrastructurally, of the relationship between the secretion granules and the nuclear and cytoplasmic constituents of the cement gland cell.

The role of the basal muscles, such as those seen in *B. eburneus, B. balanoides* and *B. amphitrite,* should be considered as an added mechanism for the distribution of cement to the basal plate region.

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SUMMARY

1. The histological characteristics of the cement gland cells of barnacles have been compared in *B. nubilis, B. psittacus, B. eburneus, B. balanoides* and *B. amphitrite.*

2. In *B. balanoides,* the cement gland cells show a very simple composition; they are situated at the base of the animal and the secretion appears scattered throughout the cytoplasm. The cement apparatus of *B. amphitrite* and *B. eburneus* looks like that of *B. tintinnabulum,* but in *B. psittacus* and *B. nubilis* the cement gland cells appear more complex.

3. The extrusion of the cement secretion is brought about by muscle fibers that pass through the connective tissue, and in *B. psittacus* and *B. nubilis* by elastic fibers around the secondary and principal canal systems.

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OBSERVATIONS ON THREE SPECIES OF JELLYFISHES FROM CHESAPEAKE BAY WITH SPECIAL REFERENCE TO THEIR TOXINS. I. *CHRYSAORA* (DACTYLOMETRA) *QUINQUECIRRHA*[1]

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Chrysaora (*Dactylometra*) *quinquecirrha*, the stinging nettle, has attracted the attention of vacationers in the Chesapeake Bay and other areas for many years because of the irritating and sometimes serious nature of its stings. At the time the present work was initiated there was no published information on the nature of the toxic agent of this species. Various terms had been applied earlier to the toxins of different Cnidaria but it is not certain that the extracted substances, *e.g.*, hypnotoxin, congestin, thallasin, *etc.* were isolated from the nematocyst capsules or that they were pure substances. Halstead (1965) has reviewed most of the important literature on the poisonous Cnidaria up to 1965.

Lane and Dodge (1958) and Lane (1960) isolated the contents of the nematocyst capsules of *Physalia physalis* (Portuguese man-of-war) and determined that these appeared to be protein in nature. Welsh (1961), page 180, states, "Much evidence indicates that the paralyzing edema-producing action of coelenterate toxins is due in large measure to a protein component(s)." Subsequently other investigators (Burnett, Stone, Pierce, Cargo, Layne and Sutton, 1968; Shapiro, 1968; Endean, Duchemin, McColm and Fraser, 1968; Crone and Keen, 1969) reach the same conclusion.

Burnett *et al.* (1968) isolated four types of nematocysts from *C. quinquecirrha* and state, page 336, "Almost all the toxic activity was localized in sediments of chemically or physically ruptured nematocyst suspensions," and conclude "that the toxin is membrane bound." However, Barnes (1967) collected the toxins of *Chironex fleckeri* and *Chiropsalmus quadrigatus* after discharge of nematocysts through a membrane (isolated human amnion) and found them to be a fluid. Endean *et al.* (1968) obtained evidence that the toxin of *C. fleckeri* is intracapsular and indicate that the capsules themselves are non-toxic.

Many of the observations reported in the present paper have been made over the past six years but the work on toxins was begun in July, 1967. This project involved (1) the extraction and isolation of the nematocyst toxins of the stinging nettle (*Chrysaora quinquecirrha*), the clover leaf jelly (*Aurelia aurita*), and the pink or lion's mane jellyfish (*Cyanea capillata*), all of which are common in the Chesapeake Bay and parts of its tributaries at certain seasons of the year; (2) toxicity experiments; and (3) the determination of the chemical nature of the toxins.

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This paper reports on the observations and experiments on the summer nettle (*C. quinquecirrha*). Research in progress with *A. aurita* and *C. capillata* will be published later.

Materials and Methods

Nettles were collected during the summer months in the Rappahannock River and Chesapeake Bay (salinity approximately 1.5%) near Deltaville, Virginia. Only the tentacles and oral lobes were used. Usually six to eight gallons of this material were processed at one time.

The following procedure for the extraction of toxin is a modification of that used by Lane and Dodge (1958) for *Physalia*. The *Chrysaora* material was allowed to autolyze in the refrigerator at 5° C for 24 to 48 hours after which it was diluted with an equal volume of 1.5% NaCl solution. This was strained through Marquisette Nylon Netting (20 openings/inch) and then through Swiss silk bolting cloth (No. 12, 125 mesh). The screened suspension was permitted to settle at 5° C in the refrigerator for 24 hours, the supernatant decanted, and discarded. The residue, consisting of nematocysts and cellular debris, was centrifuged at 7000 rpm (6000 *g*) for 15 minutes. The supernatant was discarded and an equal volume of 1.5% NaCl solution was added to the residue. The centrifuge tubes were gently shaken to loosen the cellular debris overlaying the nematocysts which firmly adhered to the bottoms of the tubes. This process was repeated several times. Each time the loosened debris was poured off. The residue was then thoroughly mixed with 1.5% NaCl solution, recentrifuged at 7000 rpm for 15 minutes, the supernatant decanted, and the above described process of washing repeated until a mass consisting almost entirely of undischarged nematocysts was secured. The whole process was continued until the supernatant was Biuret negative.

Initially 5 ml of nematocysts was mixed with 35 ml of 1.5% NaCl solution and homogenized in a Potter-Elvejhem homogenizer to disrupt the nematocysts and release their contents. However, since this was laborious and time consuming, several other methods of rupturing the capsules were tried: osmotic pressure change through the use of distilled water and ultrasonic rupture. The procedure finally adopted was that of repeated homogenization of a frozen suspension of nematocysts in a Potter-Elvejhem homogenizer. In this way 75% or more of the nematocyst capsules were ruptured or discharged, liberating the contents.

The homogenate was centrifuged in early experiments at 9000 rpm (10,000 *g*), in later ones at 18,500 rpm (40,000 *g*), for 1 hour. Most of the strongly Biuret positive supernatant was carefully pipetted off and frozen until used in toxicity and electrophoresis experiments. The residue, consisting of undischarged nematocysts, large amounts of capsular debris, and tubes was repeatedly washed in 1.5% NaCl solution and repeatedly centrifuged until the wash solution was Biuret negative. This residue was frozen for later use.

Some of the supernatant after centrifugation at 9000 rpm was pipetted off and recentrifuged at 18,500 rpm for 1 hour. The small amount of residue, consisting of microscopically fine particulate matter, was used in one of the toxicity experiments. The supernatant was treated with ammonium sulfate, centrifuged at 7000 rpm for 15 minutes, and the resulting pellet was dialyzed in standard dialysis tubing. The dialysate was used in one of the experiments.

In all toxicity experiments materials for injection were made up in 0.9% NaCl solution and 0.9% NaCl solutions were also used as controls. In all cases 1 ml doses were introduced intraperitoneally into white Swiss mice (Wistar strain).

The following standard chemical tests were used: Biuret, ninhydrin, Molisch, and Benedict's. Van Gieson's picrofuchsin and Mallory's aniline blue stains were applied to nematocysts.

Polyacrylamide gels were used to obtain the electrophoresis pattern of the toxic supernatant. The procedure for preparation of the polyacrylamide gel was suggested by the Canal Instrument Corporation in their instructions for the Model 12 Electrophoresis Apparatus. The sample and stacking gels were prepared using a buffer solution of pH 6.8–7.0; the separating gel was prepared using a buffer of pH 8.8–9.0. At the anode and cathode the pH of the buffer was 8.2–8.4.

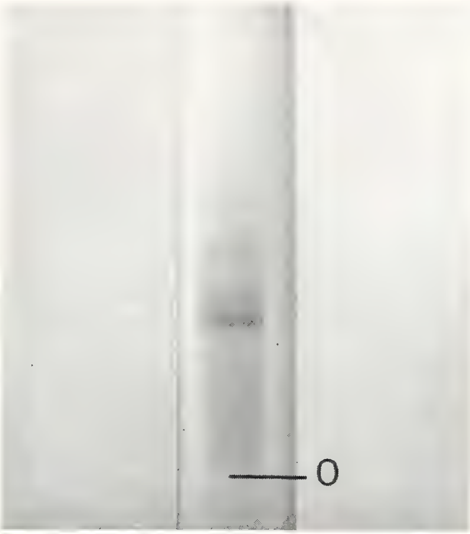
RESULTS

Over the past seven years it has been observed that contacts of different individuals with the summer nettle produce effects varying from a very faint burning sensation, barely detectable, and erythema to a severe development of angry red wheals accompanied by systemic symptoms such as nausea, respiratory distress, *etc.* On numerous occasions live nettles were deliberately permitted to come in contact with the skin of the biceps area of the arm. In some cases this produced only the faintest stinging sensation with little or no accompanying or subsequent erythema. Many individuals, however, generally showed a somewhat more severe allergic reaction.

The present study revealed four types of nematocysts. Batteries of holotrichous isorhizas (several sizes) and larger atrichous isorhizas are distributed fairly evenly over the exumbrella, oral lobes, and tentacles. The capsules of the holotrichous isorhizas range in length from 8 to 21 μ and in width from 3 to 18 μ. The tubes of these when discharged measured from 200 to 500 μ in length, 0.5 μ in diameter. The capsules of the atrichous isorhizas range from 20 to 25 μ in length and are about 15 μ in width. Most of the tubes when discharged measured from 500 to 2000 μ in length and from 0.5 to 1.5 μ in diameter. The euryteles have capsules which range from 10 to 12 μ in length and 5 to 6 μ in width. The tubes measure 50 to 150 μ in length and approximately 0.5 μ in width. The butt is 10 to 12 μ in length and approximately 1.5 μ in width. A fourth type has a round capsule 15 to 18 μ in diameter with tube length of 150 to 250 μ and diameter of 2 μ. Additional round nematocysts were observed with a diameter of 4 to 8 μ which may be smaller forms of the fourth type.

Various methods were tried in order to effect discharge of nematocysts from both living nettles and also isolated undischarged nematocysts. Immersion in distilled water and 1 *M* sucrose were ineffective in either case. Discharge of large numbers of nematocysts from the tentacles of living nettles occurred when formalin-acetic-alcohol fixative (FAA) or faradic electric shocks were applied, but neither of these agents caused the discharge of isolated nematocysts.

Since experiments on human skin *in situ* seemed out of the question, in order to get some idea of the penetration power of nematocysts the following experiment was set up: Two per cent pure agar containing enough Grenacher's borax-carmine to impart a red color was poured to a depth of about one-fourth inch in Petri dishes. Several slots, each about one-half inch long and one-eighth inch wide were cut out of each of these plates. A short piece of live tentacle was placed in each slot on the bottom against one side wall. A drop of FAA was applied to effect discharge of the nematocysts. The plates were then inverted under the microscope. The colorless nematocyst tubes could easily be seen in the red agar and their lengths measured from agar wall to tip of the tubes. Well over a hundred such measurements showed that the majority of nematocysts penetrated the agar from 200 to 400 μ. Most of the tubes took an almost straight course through the agar.



LABORATORY
LIBRARY

FIGURE 1. Electrophoretogram of the toxin of *Chrysaora* nematocysts obtained using polyacrylamide gels with subsequent staining with Coomassie blue stain for protein.

A large number of experiments dealing with the toxicity of nematocyst constituents to mice were performed as described under Materials and Methods. The results of these experiments show that the supernatant, after centrifugation of homogenized nematocyst suspensions at 9000 and 18,500 rpm, contained the toxin. The toxin is non-dialyzable, retaining its toxicity after salting out and subsequent dialysis. The LD 50, based on air dried residues corrected for salt content, was determined in three separate experiments to be 19 μg/g, 15 μg/g, and 16 μg/g of body weight, respectively. The ages of the doses of supernatant were 47, 85, and 193 days, respectively. It appears from a comparison of these figures that storage in a frozen state has little effect on toxicity. The residue obtained after removal of salt and after freeze-drying the supernatant was found to kill mice when administered at a level of 7.5 μg/g of body weight. Washed undischarged capsules, capsular debris, and tubes had no effect on mice; even the fine sediment after centrifugation at 18,500 rpm was non-toxic.

Various chemical tests were applied to undischarged nematocysts, capsular and tube debris, supernatant, and residue following centrifugation. The tests indicate that: (1) thoroughly washed capsular and tube debris are negative to ninhydrin, Biuret, Molisch, and Benedict's tests before and after boiling; (2) the capsular contents (supernatant) are ninhydrin and Biuret positive but give negative results with Molisch and Benedict's reagents; (3) the residue following centrifugation at 18,500 rpm is negative to all of the tests; (4) the fluid contents of undischarged nematocysts are Biuret positive; (5) the capsules, capsular debris, and tubes do not stain appreciably, if at all, with aniline blue or picrofuchsin.

Supernatant containing toxin was thawed from the frozen state and subjected to electrophoresis using polyacrylamide gel and subsequently stained with Coomassie blue stain for protein. The resulting electrophoretogram is shown in Figure 1. One main band can be seen indicating one major protein fraction. Three faint bands may also be observed. Repetition using the supernatant from nematocysts obtained from another collection of nettles confirmed this picture.

Discussion

Burnett *et al.* (1968) observed four types of nematocysts from *Chrysaora*: Type I, oval atrichous isorhizas; Type II, round structures; Type III, euryteles; Type IV, small round structures. Halstead (1965) in a table adapted from several authors records three types of nematocysts from *Chrysaora*: atrichous isorhizas, holotrichous isorhizas, and heterotrichous microbasic euryteles. The last type is followed by a question mark indicating uncertainty. In general our observations agree with those of Burnett *et al.* (1968) except that they do not record holotrichous isorhizas. The euryteles observed by us do not appear to be heterotrichous microbasic euryteles but probably fit better in the eurytele class than any other.

Several methods for causing nematocyst discharge from living nettles have been used. Kline and Waravdekar (1960) used electric shock to effectively produce discharge of nematocysts of *Hydra littoralis*. More recently Barnes (1967) employed electrical shock with success on *C. fleckeri*, *C. quadrigatus* and *Cyanea capillata*. We have found that faradic shocks are quite effective over small areas of the tentacles of *Chrysaora*. FAA fixative gave good results but distilled water and 1 *M* sucrose were of little value.

Phillips and Abbott (1957) effected discharge of isolated nematocysts of *Metridium senile fimbriatum* by subjecting this anemone to various chemical agents: distilled water; methylene blue; weak acids; weak bases; sucrose; and glycerine. Burnett *et al.* (1968) compared the effectiveness of a number of physical and chemical agents in producing nematocyst rupture (not discharge) in *Chrysaora*, among them grinding; freeze thaw; distilled water; salts, acids; bases, cholinergic and adrenergic drugs; heat; and sonication. In contrast with the above results we were able to observe little or no discharge or rupture with distilled water, 1 *M* sucrose, FAA fixative, or faradic shock. Sonication of suspensions of nematocysts resulted in somewhat better than 50% rupture. The homogenization of frozen nematocyst suspensions, the procedure finally adopted, was found to be much more efficient than the method of Lane and Dodge (1958), yielding as high as 75% rupture.

Our studies of the penetration of nematocyst tubes into agar are the first to be made with *Chrysaora*. However, Cleland and Southcott (1965) record some work on the discharge of nematocysts of Australian species of jellyfishes into human skin and the pathological effects. Barnes (1967) made observations on the penetration of nematocysts of *Chironex*, *Chiropsalmus* and *Cyanea* into various materials: human amnion; sheep intestine; hog stomach; fish swim bladders; synthetic sausage casings; latex; cured rubber; cellulose and various polyethylene and polyvinyl films. Further investigations in addition to those noted here should give much useful information for different species of cnidarians relative to the force of discharge of nematocysts, depth of penetration of tubes and the paths taken in test materials of various kinds.

As stated earlier in this paper a number of investigators (Lane and Dodge, 1958; Lane, 1960; Welsh, 1961; Burnett *et al.* 1968; Shapiro, 1968; Endean *et al.* 1968; Crone and Keen, 1969) have found the toxins of various cnidarians to be protein in nature. Burnett *et al.* (1968) believe (p. 335) that "the toxin factor of *Chrysaora* is a protein complex or is associated with a protein." The present work leaves little doubt that the toxin of *Chrysaora* is a protein or several proteins.

Burnett *et al.* (1968) state further that after nematocyst rupture (p. 335) "the toxin can be recovered in significant amounts in the sediments," . . . (p. 336) "that the toxin is membrane bound," . . . and (p. 335) "that the toxin is not released as a free fluid after nematocyst rupture." In contrast, the results of the present experiments show that the toxin is (1) contained in the undischarged nematocysts and is released as a free fluid after rupture which agrees with the findings of Barnes (1967) and Endean *et al.* (1968) for other species of jellyfishes; (2) the toxin is not membrane bound (that is, to the capsules); and (3) the toxin is not present in thoroughly washed empty capsules, capsular fragments, tubes, nor in sediments after centrifugation at 9000–18,500 rpm which Endean *et al.* (1968) also indicate is true of the nematocysts of *C. fleckeri*. The differences in the findings of Burnett *et al.* (1968) and the results obtained in the present work might be due to differences in procedures followed in the isolation of the toxin.

Our LD 50 determinations in general agree with those of Burnett *et al.* (1968). They conclude, however, that toxicity loss resulted from freezing refrigeration of nematocysts. The LD 50 values reported in the present work for three separate experiments indicate that storage in the frozen state for better than six months had little effect on the toxicity of nematocyst contents.

It has been suggested by several investigators, Lenhoff, Kline and Hurley (1957), Phillips (1956), Kline (1961), Goldher, Burnett, Stone and Dilaimy (1969), that the capsules of various Cnidaria are composed of, or contain, a protein of the collagenous group. If this is so, *Chrysaora* material would appear to be different from vertebrate collagen since the capsules do not stain with aniline blue nor picrofuchsin which are accepted stains for vertebrate collagen.

We acknowledge with thanks the assistance of the following persons: Mr. William A. Dorsey, Chief of Public Laboratories, Richmond City Health Department for supplying us with laboratory mice; Mr. James Rose, Research Chemist, Department of Surgery, Health Sciences Division, Virginia Commonwealth University, Richmond, for electrophoresis studies; Dr. Wilton R. Tenney, Department of Biology, University of Richmond for the photograph of the electrophoretogram;

and Dr. Francis B. Leftwich, Department of Biology, University of Richmond for technical assistance.

SUMMARY

1. The response of the human body to the toxin of the summer nettle, *Chrysaora quinquecirrha,* is allergic in nature.

2. Four types of nematocysts were identified: atrichous isorhizas; holotrichous isorhizas; euryteles; and a round type.

3. Application of faradic electrical shocks and the fixative FAA were effective in producing discharge of nematocysts in living nettles but not of isolated nematocysts.

4. The tubes of freely discharged nematocysts measured from 500–2000 μ in length. When discharged into 2% agar, the majority of tubes penetrated a distance of only 200–400 μ.

5. An efficient method of isolation and rupture of the nematocysts with subsequent isolation of the toxin is described.

6. The toxin is non-dialyzable. It gives positive ninhydrin and Biuret tests but negative Molisch and Benedict's tests.

7. The toxin is a protein or several proteins and is contained in the free fluid discharged from the nematocyst capsules. It is not membrane bound.

8. When inoculated intraperitoneally into white mice, the toxin gave an LD 50 of 19 μg/g, 15 μg/g, and 16 μg/g in three experiments.

9. There is little if any loss in toxic activity of nematocyst contents after storage in the frozen state over a six-month period.

10. The toxicity of the supernatant is retained after freeze-drying.

11. Thoroughly washed empty capsules, capsular fragments, and tubes do not appear to have any toxic effect when injected intraperitoneally into white mice.

12. Thoroughly washed empty nematocyst capsules, capsular fragments and tubes, and residues from high speed centrifugation gave negative results when tested with ninhydrin, Biuret, Molisch, and Benedict's reagents.

13. Empty washed capsules, capsular debris, and tubes do not stain with aniline blue nor picrofuchsin which are accepted stains for vertebrate collagen.

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LARVAL DEVELOPMENT OF *PAGURUS LONGICARPUS* SAY REARED IN THE LABORATORY, I. DESCRIPTION OF LARVAL INSTARS[1]

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Thompson (1903) described from the plankton four zoeae and a megalopa which he ascribed to *Pagurus longicarpus*. Although his verbal description of the external anatomy is incomplete, his figures are adequate to identify accurately the species with which he was working. His description of the internal anatomy is the most complete study available for any decapod larva. He indicated that some larvae of *P. annulipes* were included in his study material but that he was unable to differentiate the two species.

MacDonald, Pike, and Williamson (1957) and Pike and Williamson (1959, 1960) described the larvae of several British and Mediterannean *Pagurus* species from carefully constructed plankton series. They were able to distinguish three types of larvae in this genus, based on 12 larval characteristics (several of which were composites of two characteristics), suggesting that the genus *Pagurus* may be polyphyletic, but no division of the genus was attempted for lack of adult characteristics supporting the larval data.

Larvae of several additional *Pagurus* species have been described from culture experiments by Coffin (1958, 1960), Hart (1937), Provenzano and Rice (1964) and Shenoy (1967). In addition, *P. bernhardus* has been cultured by Bookhout (1964) and A. Le Roux (personal communication) and shown to agree in all essentials with the description given by MacDonald *et al.* (1957).

This paper deals with the external anatomy of *P. longicarpus* larvae reared in the laboratory. It is clearly demonstrated that Thompson (1903) attributed his larvae to the wrong species and a suggestion is made as to the correct identity of his larvae.

MATERIALS AND METHODS

Ovigerous *P. longicarpus* were collected from the beach at Gloucester Point, Virginia. Some crabs were maintained at room temperature in finger bowls with filtered water (salinity: 19–22‰). The water was replenished once or twice daily until hatching occurred. This method had two disadvantages: first, there was a danger of hatching occurring without molting the embryonic or prezoeal cuticle; second, adult hermit crabs kept in small containers eat many larvae before they can be removed. These problems were overcome in some experiments by removing the eggs from the pleopods of the crabs and culturing the eggs artificially (Costlow and Bookhout, 1960).

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Larvae were cultured individually in compartmented plastic boxes with nauplii of *Artemia salina* as food. No bactericide was used and water was changed daily. Living specimens of each instar were preserved in 70% ethanol with 10% glycerol as were exuvia and dead specimens. Live specimens proved most satisfactory for study as they were less likely to be damaged and easier to handle and dissect.

Several larvae in each instar were dissected under 85% lactic acid and mounted in a modified Hoyer's medium for study and figuring. Figures of entire specimens and various appendages were drawn with the aid of an ocular grid and graph paper. Verbal descriptions, prepared from figures and notes taken during study, were subsequently checked against additional specimens. Notes on color were made while observing living larvae immobilized mechanically as suggested by Dean and Hatfield (1963). Larvae handled in this manner survived when returned to the culture dish and showed no obvious deleterious effects.

The following abbreviations are used throughout the description: A 1 = antennule, A 2 = antenna, Mn = mandible, Mx 1 = maxillule, Mx 2 = maxilla, Mxp 1 = first maxilliped, Mxp 2 = second maxilliped, Mxp 3 = third maxilliped, P 1 to 5 = pereiopods 1 to 5, Pl 2 to 5 = pleopods 2 to 5, U = uropods.

RESULTS

Four zoeal stages and a megalopa were obtained. No prezoea was observed except when culture conditions for the eggs were known to be suboptimal. Examination of egg membranes revealed that the embryonic or prezoeal cuticle normally is shed at the time of hatching and remains attached to the egg membrane. It is concluded that the prezoea is not a true planktonic stage and therefore no description is given.

Zoea I (Figure 1)

Rostrum unornamented, curved slightly ventrad, approximately equal to A 1 and A 2. Carapace with small posterolateral spines pointed ventrally. Eyes unstalked. Abdomen with 5 somites plus fused 6th somite-telson. Somites 2 through 5 with 2 pairs of posterodorsal spines and one pair of ventrolateral spines, those of somite 5 longest, reaching fusion of 6th somite-telson. Pleopod buds and uropods absent. Telson formula *7 + 7*; process 1, lateral spur; process 2, short ventral hair; processes 3 to 7, long articulated plumose setae, process 4 longest. Fine hairs between processes. Small median notch. Anal spine present.

A 1 (Fig. 1C)—Uniramous, unsegmented, with 4 terminal aesthetascs and a long subterminal plumose seta.

A 2 (Fig. 1D)—Scale with 8 (7–9) plumose setae on inner margin and terminal spur. Endopod about $\frac{1}{2}$ length of scale, fused to peduncle, with 2 terminal plumose setae. Short strong seta with cuneate setules on peduncle near endopod.

Mn (Fig. 1E)—Incisor and molar processes present, palp absent.

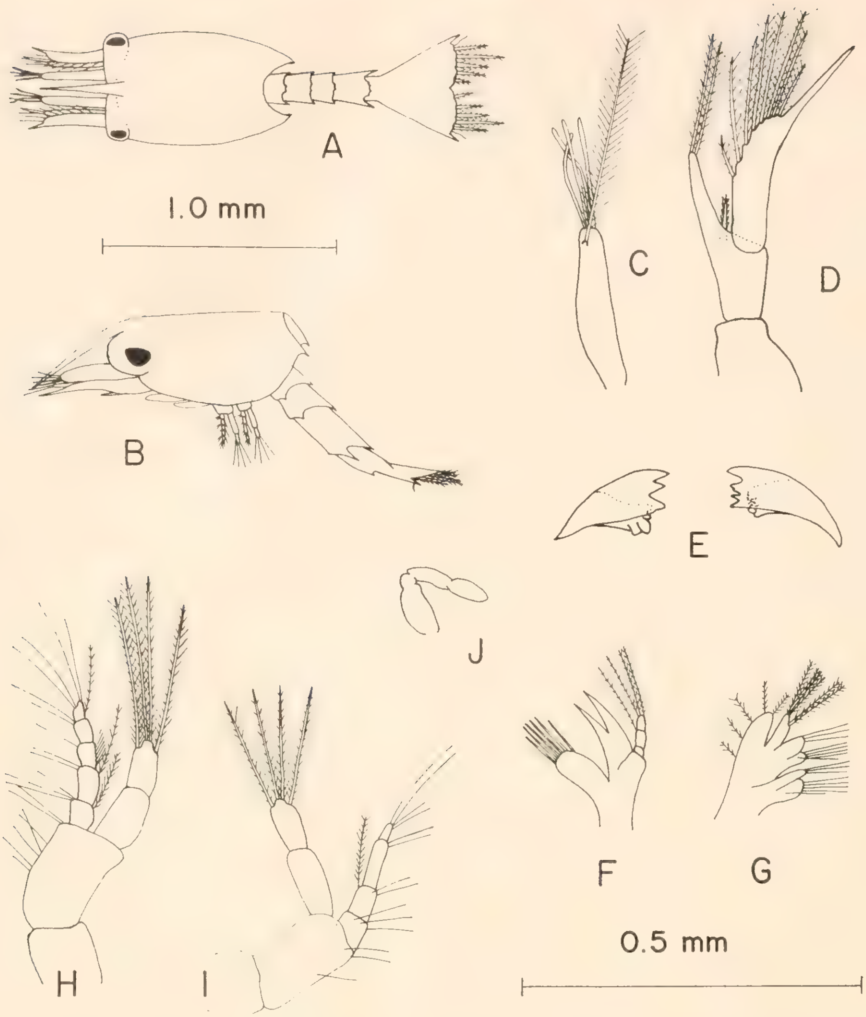

Figure 1. Zoea I of *Pagurus longicarpus;* A. dorsal view, B. lateral view, C. antennule, D. antenna, E. mandibles, F. maxillule, G. maxilla, H. 1st maxilliped, I. 2nd maxilliped, J. 3rd maxilliped.

Mx 1 (Fig. 1F)—Coxal endite with 6 setae, basal endite produced into 2 strong spines. Endopod 3-segmented with 3 terminal plumose setae and an additional seta distomedially on second segment.

Mx 2 (Fig. 1G)—Coxal and basal endites bilobed, both with 5 setae on proximal lobe, 3 on distal lobe. Endopod unsegmented with 4 terminal and 2 subterminal setae. Scaphognathite with distal lobe bearing 5 marginal plumose setae.

Mxp 1 (Fig. 1H)—Coxa nude, basis with 7 setae on inner margin. Endopod 5-segmented: long plumose seta on outer margin of segments 1, 2, and 5; fringe of fine hairs on outer margin of segment 3; 3, 2, 1, 2, 4 setae on inner margin of segments 1 to 5. Exopod 2-segmented, with 4 terminal plumose setae.

Mxp 2 (Fig. 1I)—Coxa nude, basis with 3 setae on inner margin. Endopod 4-segmented: long plumose seta on outer margin of segment 2; 3, 2, 2, 4 setae on inner margin of segments 1 to 4. Exopod 2-segmented, with 4 terminal plumose setae.

Mxp 3 (Fig. 1J)—Uniramous unsegmented rudiment, flexed anteriorly and medially between bases of Mxp 2.

P 1 to 5 (Fig. 1B)—Barely distinguishable rudiments initially but noticeably larger by end of stage.

Pigmentation—Red chromatophore midway along rostrum, few if any chromatophores on rest of carapace at hatching, several pairs developing during this stage near posterior margin. Two red chromatophores in each eyestalk region. Red chromatophores on base of A 2, on all inner mouth parts, encircling labrum, and 2 red and yellow chromatophores on basis of Mxp 1, 1 on Mxp 2. Chromatophores sometimes in proximal segment of exopod of Mxp 1 and 2. Chromatophores in intestine in somites 2 and 4; 6th somite-telson with pair of red and 3 pairs of red-yellow chromatophores. In most specimens, a pair of red or yellow chromatophores located at posterolateral corners of telson. Pigmentation variable.

Zoea II (Figure 2)

Rostrum slightly longer than A 1 and A 2. Eyes stalked. Abdomen with 5 somites and fused 6th somite-telson. Pleopod buds and uropods absent, but uropod anlagen visible within telson late in stage. Telson formula 8 + 8; process 8 added medially to process 7; process 4 still articulated and longest. Anal spine present.

A 1 (Fig. 2C)—No change from Zoea I.

A 2 (Fig. 2D)—Slight increase in size; endopod articulated to peduncle.

Mn (Fig. 2E)—No change from Zoea I.

Mx 1 (Fig. 2F)—Coxal endite with 7 setae, basal endite with 4 spines and a small seta, spines articulated. Endopod with 3 terminal plumose setae and 1 plumose seta on inner margin of second segment.

Mx 2 (Fig. 2G)—Coxal and basal endites bilobed. Coxal endite with 7 setae on proximal lobe, 4 on distal lobe. Basal endite with 5 setae on proximal lobe, 4 on distal lobe. Endopod with 3 terminal, 1 subterminal, and 2 (3) medial plumose setae. Scaphognathite with distal lobe bearing 8 plumose setae.

Mxp 1 (Fig. 2H)—Coxa with 1 seta, basis with 10 setae on inner margin. Endopod: long plumose seta on outer margin of segments 1, 2, 3, and 5; 3, 2, 1, 2, 4 setae on inner margin of segments 1 to 5. Exopod with 7 terminal plumose setae.

Mxp 2 (Fig. 2I)—Coxa nude, basis with 3 setae on inner margin. Endopod: long plumose seta on outer margin of segments 2, 3, and 4; 3, 2, 2, 4 setae on inner margin of segments 1 to 4. Exopod with 7 terminal plumose setae.

Mxp 3 (Fig. 2J)—Biramous rudiment. Endopod bud fused to basis; 2 terminal setae. Exopod 2-segmented, with 6 terminal plumose setae, functional for swimming.

P 1 to 5 (Fig. 2B)—Small uniramous buds increasing in length somewhat during stage. Occasionally pseudosegmented at end of stage.

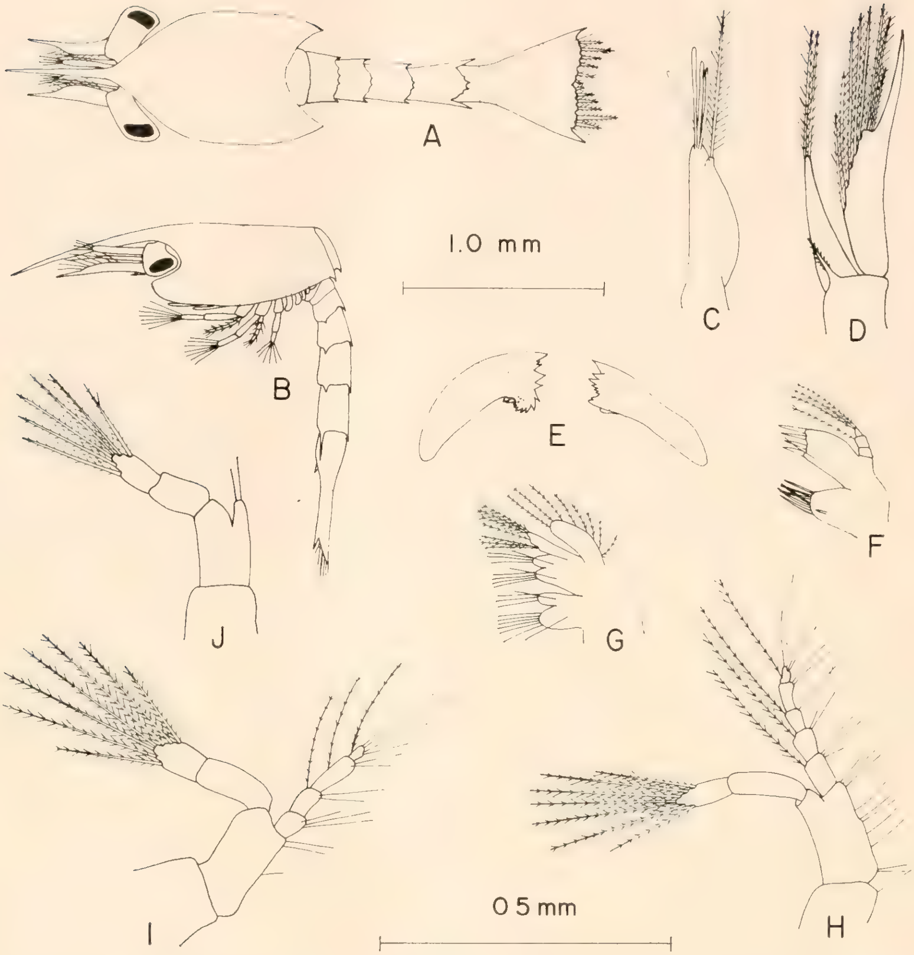

Figure 2. Zoea II of *Pagurus longicarpus;* A. dorsal view, B. lateral view, C. antennule, D. antenna, E. mandibles, F. maxillule, G. maxilla, H. 1st maxilliped, I. 2nd maxilliped, J. 3rd maxilliped.

Pigmentation—Generally as in Zoea I but more extensive. Eyestalks with red and yellow chromatophores. Carapace with additional chromatophores on lateral and dorsal regions. Mouth parts pigmented as in Zoea I. Red and yellow chromatophores developing dorsally in abdominal somite 2. Pattern on carapace extremely variable.

Zoea III (*Figure 3*)

Rostrum and carapace unchanged. Abdominal somite 6 free from telson, with smooth posterior margin. Ventrolateral spines on somite 5 projecting posteriorly to about middle of somite 6. Telson formula 8 + 8 as in Zoea II; process 4 fused to telson, greater than $\frac{1}{2}$ maximum telson width.

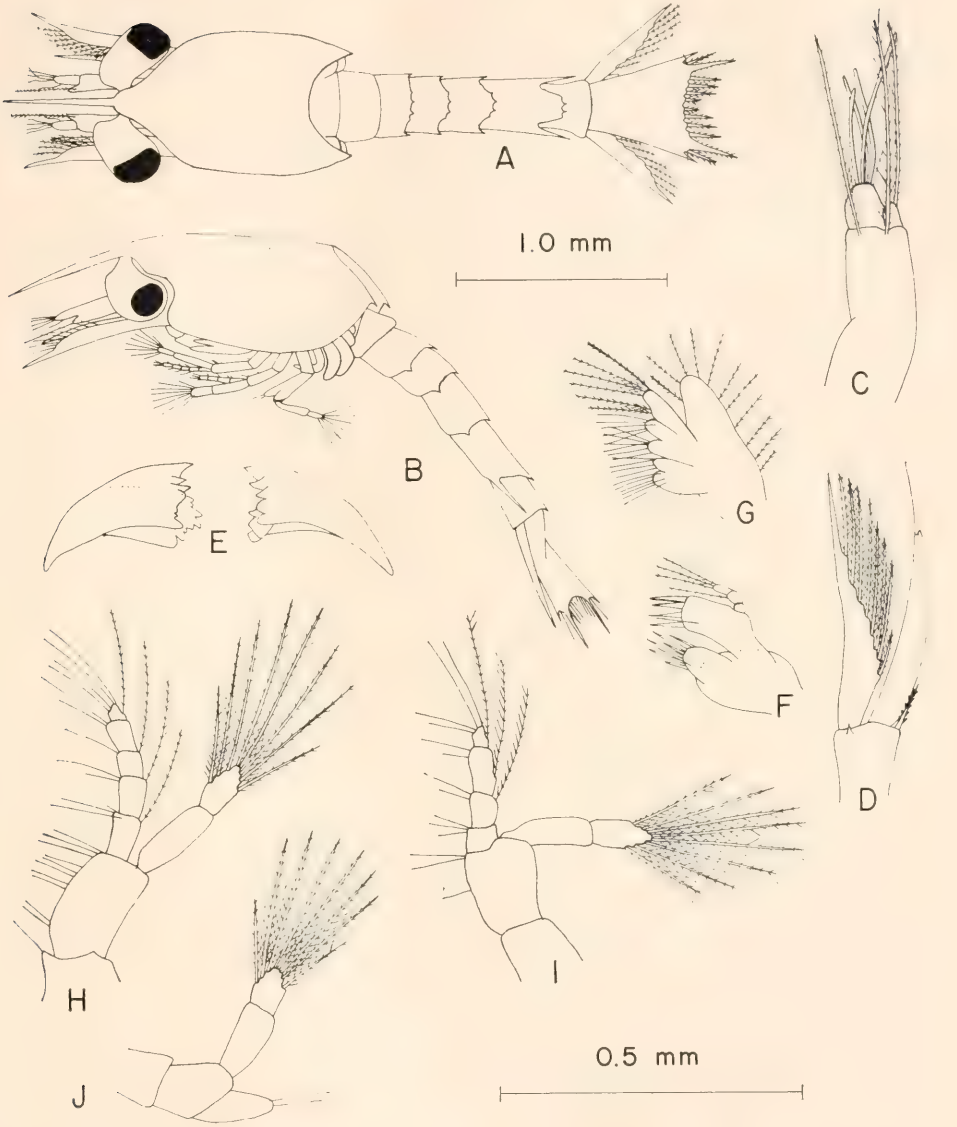

FIGURE 3. Zoea III of *Pagurus longicarpus;* A. dorsal view, B. lateral view, C. antennule, D. antenna, E. mandibles, F. maxillule, G. maxilla, H. 1st maxilliped, I. 2nd maxilliped, J. 3rd maxilliped.

A 1 (Fig. 3C)—Biramous, with both rami unsegmented and articulated to peduncle. Outer ramus with 4 terminal aesthetascs plus several short setae; inner ramus nude. Three long plumose setae on distal end of peduncle, 1 near articulation of outer ramus and 1 on each side of inner ramus.

A 2 (Fig. 3D)—Scale with 9 (8) plumose setae. Endopod equal to scale minus terminal spur, with 1 (2) terminal setae. Ventral seta present as in Zoea I and II plus a short spine added ventral to scale.

Mn (Fig. 3E)—No change from Zoea I.

Mx 1 (Fig. 3F)—Coxal endite with 7 setae, basal endite with 4 spines and a small seta, spines articulated. Endopod with 3 terminal plumose setae and 1 plumose seta on inner margin of second segment.

Mx 2 (Fig. 3G)—Coxal and basal endites bilobed. Coxal endite with 7 setae on proximal lobe, 4 (3) on distal lobe. Basal endite with 6 setae on proximal lobe, 5 on distal lobe. Endopod with 4 terminal and 2 subterminal plumose setae. Distal lobe of scaphognathite with 11 plumose setae.

Mxp 1 (Fig. 3H)—Coxa with 1 seta, basis with 10 setae on inner margin. Endopod: long plumose seta on outer margin of segments 1, 2, 3 and 5; 3, 2, 1, 2, 4 setae on inner margin of segments 1 to 5. Exopod with 8 terminal plumose setae.

Mxp 2 (Fig. 3I)—Coxa nude, basis with 3 setae on inner margin. Endopod: long plumose seta on outer margin of segments 2, 3 and 4; 3, 2, 2, 4 setae on inner margin of segments 1 to 4. Exopod with 8 terminal plumose setae.

Mxp 3 (Fig. 3J) Coxa and basis both nude. Endopod unsegmented, articulated with basis, with 1 terminal and 1 subterminal seta. Exopod with 8 terminal plumose setae.

P 1 to 5 (Fig. 3B)—Increase considerably in size during stage, pseudosegmented at end of stage. P 5 hidden behind P 1 to 4, not readily visible.

U (Fig. 3A, B)—Freely articulated, biramous; exopod $\frac{3}{4}$ telson length with 6 plumose setae on inner margin plus terminal spur; endopod rudimentary.

Pigmentation—Generally as in Zoea II. Chromatophores of abdominal somite 6 spilling over into somite 5 and telson. Small red and yellow chromatophores beginning to develop in pereiopod buds.

Zoea IV (*Figure 4*)

Carapace, abdominal somites, and telson as in Zoea III.

A 1 (Fig. 4C)—Peduncle with 2 short rami. Outer ramus unsegmented with 4 terminal aesthetascs and 5 (6) aesthetascs on inner margin, plus several fine terminal setae. Inner ramus unsegmented, nude. Three long plumose setae on distal end of peduncle as in Zoea III.

A 2 (Fig. 4D)—Scale with 9 (7–9) plumose setae and terminal spur. Endopod longer than scale, reaching tip of rostrum. Initially unsegmented, but segments evident beneath cuticle just prior to molt; appears folded like an accordion.

Mn (Fig. 4E)—Unsegmented palp rudiment present. Molar and incisor processes unchanged.

Mx 1 (Fig. 4F)—Coxal endite with 8 (7–9) setae, basal endite with 7 (6–8) spines. Endopod with 3 terminal plumose setae plus 1 plumose seta at distal end of second segment.

Mx 2 (Fig. 4G)—Coxal and basal endites bilobed. Coxal endite with 7 (7–8) setae on proximal lobe, 4 (4–5) on distal lobe. Basal endite with 5 (4–6) setae on proximal lobe, 6 (4–6) on distal lobe. Endopod with 4 long terminal plumose setae and 3 (4) plumose setae midway along inner margin. Scaphognathite with

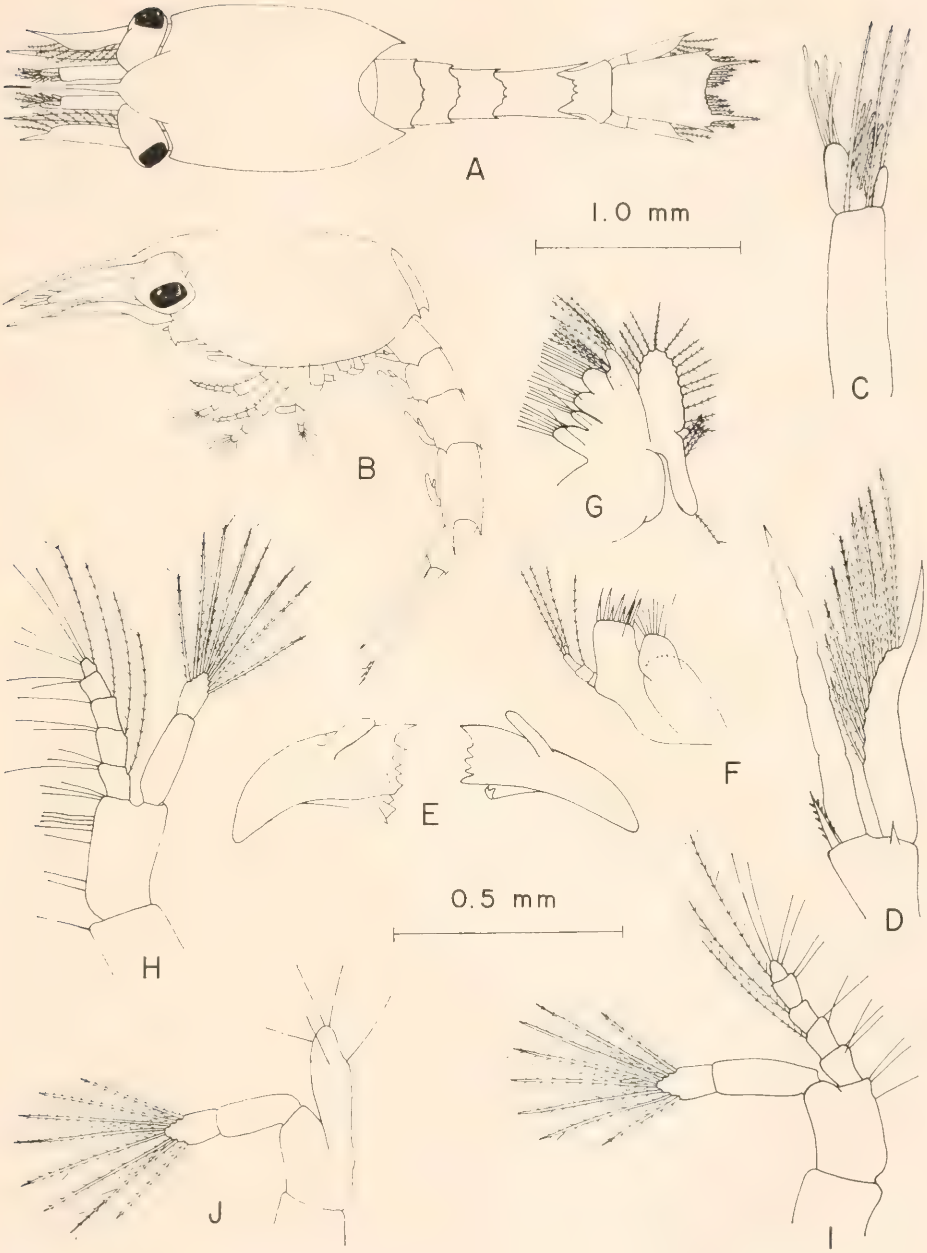

FIGURE 4. Zoea IV of *Pagurus longicarpus;* A. dorsal view, B. lateral view, C. antennule, D. antenna, E. mandibles, F. maxillule, G. maxilla, H. 1st maxilliped, I. 2nd maxilliped, J. 3rd maxilliped.

distal and proximal lobes present, with 18 (11–20) plumose setae. Lobes of equal length; proximal only $\frac{1}{2}$ width of distal.

Mxp 1 (Fig. 4H)—Coxa with 1 seta, basis with 10 (10–12) setae on inner margin. Endopod: long plumose seta on outer margin of segments 1, 2, 3 and 5; 3, 2, 1, 2, 4 setae on inner margin of segments 1 to 5. Exopod with 8 terminal plumose setae.

Mxp 2 (Fig. 4I)—Coxa nude, basis with 3 setae on inner margin. Endopod 5-segmented, unlike previous stages; long plumose seta on outer margin of segments 2, 3 and 5; 3, 2, 0, 2, 4 setae on segments 1 to 5. Exopod with 8 terminal plumose setae.

Mxp 3 (Fig. 4J)—Coxa and basis nude. Endopod short, pseudosegmented, with a few scattered setae distally. Exopod with 8 terminal plumose setae.

P 1 to 5 (Fig. 4B)—Segmented; P 1 cheliform, others styliform. Increase considerably during stage until space beneath carapace filled, but remain flexed against cephalothorax.

Pl 2 to 5 (Fig. 4B)—Pleopod buds paired on somites 2 to 5. Biramous, with endopod minute, nude, inconspicuous. Exopod usually nude, rarely with 5 terminal setae. Buds increase in length by about twice during stage.

U (Fig. 4A, B)—Exopod with 6 plumose setae on inner margin and 1 (2) terminal spurs, approximately equal to telson (exclusive of telson processes). Endopod still rudimentary but longer, with 2 terminal setae.

Pigmentation—As in Zoea III, although more conspicuous on carapace. Abdominal somite 5 and telson increasingly pigmented. Mxp 3 with a red-yellow chromatophore in basis. Pereiopod buds with large chromatophores. As in previous stages, pattern extremely variable even among larvae from a single female.

Megalopa (*Figure 5*)

Carapace (Fig. 5A) with no posterolateral spines, rostrum greatly reduced and broadly rounded as in adult. Eyes stalked, peduncle slightly swollen in middle; eye scales present. Abdomen with 6 discrete segments and telson, slightly asymmetric in many specimens. Telson (Fig. 5A, P) with 4 + 4 terminal plumose setae and a minute lateral spine.

A 1 (Fig. 5C)—Outer ramus 4-segmented with 3 aesthetascs on second segment, 4 on third and proximally on last segment, terminated with 1 long and 3 short setae. Inner ramus 2-segmented with a few short setae.

A 2 (Fig. 5B)—Flagellum with 10 to 14 segments, all with short setae distally. Scale reduced, unidentate, with a few scattered setae.

Mn (Fig. 5D)—Of adult shape. Palp 3-segmented with a few setae on last segment.

Mx 1 (Fig. 5E)—Coxal endite with short setae, basal endite with 3 rows of 5 teeth each. Endopod unsegmented, no palp.

Mx 2 (Fig. 5F)—Coxal and basal endites bilobed with numerous short setae. Endopod unsegmented with 3 terminal and 1 subterminal setae. Scaphognathite with 32–35 setae.

Mxp 1 (Fig. 5G)—Coxal endite small, not completely formed, with 4 setae. Basal endite considerably larger, but also not of adult shape, with many short setae. Endopod slightly reduced, unsegmented, with few setae. Exopod 2-seg-

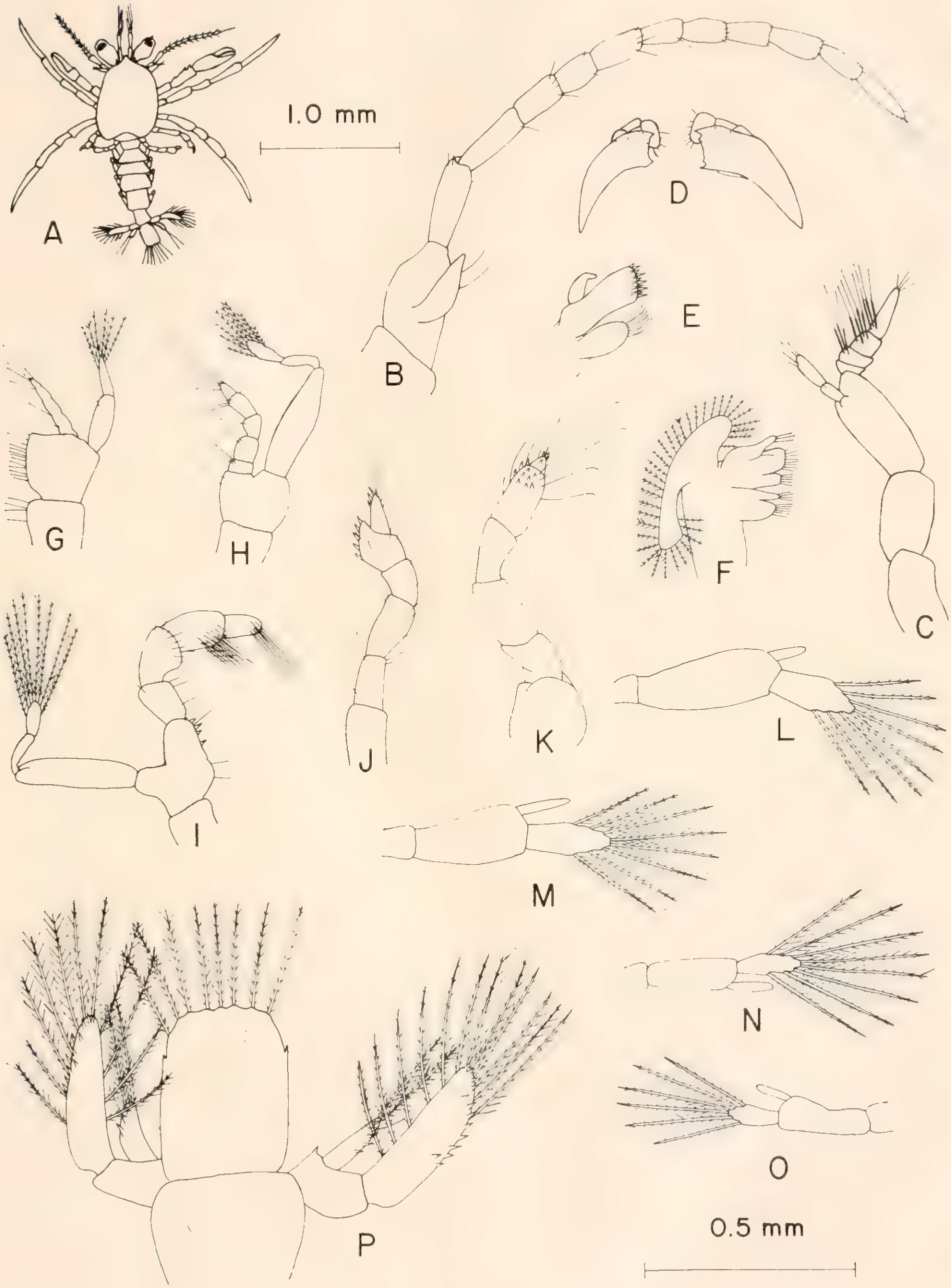

FIGURE 5. Megalopa of *Pagurus longicarpus;* A. dorsal view, B. antenna, C. antennule, D. mandibles, E. maxillule, F. maxilla, G. 1st maxilliped, H. 2nd maxilliped, I. 3rd maxilliped, J. 4th pereiopod, K. 5th pereiopod, L. 2nd pleopod, M. 3rd pleopod, N. 4th pleopod, O. 5th pleopod, P. telson and uropods.

mented with proximal segment still narrow and lacking marginal setae. Distal segment with 6 plumose setae.

Mxp 2 (Fig. 5H)—Coxa nude. Basis with 2 setae on inner margin. Endopod 5-segmented, with fewer setae than adult. Exopod 3-segmented, with 8 terminal plumose setae.

Mxp 3 (Fig. 5I)—Coxa nude. Endopod 5-segmented; basis fused with ischium; dentate ridge with 3 (4) teeth plus accessory tooth and 4 setae; all segments with fewer setae than adult. Exopod 3-segmented, with 8 terminal plumose setae.

P 1 to 5 (Fig. 5A, J, K)—P 1 cheliform, slightly asymmetric, right larger than left, reaching A 2. P 2 and P 3 of adult proportions, functional as ambulatory legs. P 4 (Fig. 5J) subchelate, propodus with a single row of 5 tubercles representing adult rasp, dactylus with 3 tubercles. P 5 (Fig. 5K) with 10 tubercles arranged in several rows and several hooked setae on propodus, 3 tubercles and several hooked setae on dactylus.

TABLE I

Carapace and total length (in mm) of Pagurus longicarpus *and* Pagurus annulipes *larvae*

		Zoea				Megalopa
		I	II	III	IV	
Pagurus longicarpus						
Carapace	X	1.05	1.23	1.47	1.66	1.13
Length	range	0.99–1.10	1.12–1.40	1.35–1.64	1.44–1.80	0.90–1.26
	N	20	27	35	35	40
Total	X̄	1.74	2.40	2.90	3.38	2.39
Length	range	1.67–1.80	2.16–2.61	2.70–3.19	3.06–3.60	2.05–2.70
	N	20	26	35	32	36
*Pagurus annulipes**						
Total Length	range	1.9–2.7	2.7–3.0	3.5–4.0	4.0	2.8–3.3

* after Thompson (1903) as *P. longicarpus.*

Pl 2 to 5 (Fig. 5L, M, N, O)—Pleopods still paired on somites 2 to 5; biramous, endopod small; 8, 8, 8, 5 (6) terminal plumose setae on exopods of Pl 2 to 5, natatory.

U (Fig. 5A, P)—Left larger, with 12 (14) plumose setae and 11 (13) tubercles on exopod, 3 plumose setae and 4 (6) tubercles on endopod. Right with 11 (13) plumose setae and 9 (11) tubercles on exopod, 3 plumose setae and 3 (4) tubercles on endopod. Basis with a small spine near origin of endopod.

Pigmentation—Many more chromatophores present, but pattern essentially same as in zoeae. As next molt approaches, chelipeds become pale yellow or white as in juvenile.

Growth—The larval stages increased relatively uniformly from a total length of 1.74 mm in Zoea I to 3.38 mm in Zoea IV (Table I). With the molt to the megalopa, the total length dropped about 1 mm or 30% to approximately the size of Zoea II. Comparison of carapace lengths revealed the same result. It would be

TABLE II

Summary of anatomical differences between Pagurus longicarpus *and* Pagurus annulipes *larvae*

Stage	Pagurus longicarpus	Pagurus annulipes
Zoea I	A 2 scale with 8 setae	A 2 scale with 10 setae
	A 2 endopod $= \frac{1}{2}$ scale	A 2 endopod $< \frac{1}{2}$ scale
Zoea II	A 2 scale with 8 setae	A 2 scale with 10 setae
	Mx 2 scaphognathite with 8 setae	Mx 2 scaphognathite with 6 setae
Zoea III	A 2 scale with 9 setae	A 2 scale with 10 setae
	Mx 2 scaphognathite with 11 setae	Mx 2 scaphognathite with 10 setae
	Abdominal somite 6 without median dorsal spine	Abdominal somite 6 with median dorsal spine
	Telson process 4 long	Telson process 4 minute
Zoea IV	A 2 scale with 9 setae	A 2 scale with 8 setae*
	Mn palp present	Mn palp absent
	Mx 2 scaphognathite with 18 setae; proximal lobe present	Mx 2 scaphognathite with 11 setae; proximal lobe absent
	Mxp 2 endopod 5-segmented	Mxp 2 endopod 4-segmented
	U with 6 plumose setae	U with 7 plumose setae
	Abdominal somite 6 without median dorsal spine	Abdominal somite 6 with median dorsal spine
	Telson process 4 long	Telson process 4 minute
All Zoeae	Red chromatophores absent ventrolaterally on carapace and on abdominal somites 2 and 5	Red chromatophores present ventrolaterally on carapace and on abdominal somites 2 and 5
	Mx 1 endopod 0, 1, 3 setae	Mx 1 endopod 1, 1, 2 setae Zoea I–III; 1,1,3 setae, Zoea IV
	Mxp 1 and 2 exopod 2-segmented	Mxp 1 and 2 exopod unsegmented
	Mxp 3 exopod 2-segmented (Zoea II–IV)	Mxp 3 exopod unsegmented
	Lateral abdominal spines long	Lateral abdominal spines short
Megalopa	Mxp 1,2,3 well developed	Mxp 1,2,3 poorly developed
	$P\ 1 = A\ 2 < P\ 2$ and P3	$P\ 1 = P\ 2$ and $P\ 3 > A\ 2$
	Pl 2 to 5 with 8,8,8,5 setae	Pl 2 to 5 with 7,7,9,7 setae**

* A 2 scale with 10 setae according to C. Nyblade.

** Pl 2 to 5 with 9(10),9(10),9(10),8 setae according to C. Nyblade.

interesting to follow changes in weight through the larval sequence but this has not been done. The change in length probably reflects the radical change in shape rather than a true change in biomass or volume.

The larva described by Thompson (1903) was considerably larger than *P. longicarpus,* 0.5 mm or more at each instar (Table I). Again there was an increase in total length to Zoea IV and then a reduction to about the length of Zoea II. Thompson used the wide range of lengths within each zoeal stage to justify partially his contention that he was working with two species, but, with the exception of Zoea I, the range of total length which he observed was equal to or smaller than that observed in the present study.

DISCUSSION

The larvae described by Thompson (1903) differ from *P. longicarpus* as described above in many features, some quite conspicuous. Table II summarizes these

TABLE III

Summary of characteristics defining the four groups of Pagurus *larvae recognized with a list of the species in each group (After MacDonald et al., 1957)*

Characteristics	Group			
	A	B	C	D
Zoeal length and telson breadth	elongate narrow	not elongate broad	not elongate broad	not elongate broad
Lateral spines somite 5	long	long	medium	long
Median dorsal spine somite 6	absent	present	absent	absent
Telson process 4, Zoea III and IV	long $>\frac{1}{2}$ telson width fused	reduced $<\frac{1}{2}$ telson width fused	medium $=\frac{1}{2}$ telson width articulated	long $>\frac{1}{2}$ telson width fused
Number setae on endopod of A 2	0	2	2	2
A 2 scale	straight $L \geq 6 \times W$ setae ≤ 8	curved $L < 4 \times W$ 9 setae SI 10 setae SII–IV	curved $L < 4 \times W$ 9 setae SI 10 setae SII–IV	curved $L < 4 \times W$ 8 setae SI–II 9 setae SIII–IV
Mandibular palp, Zoea IV	present	absent	absent	present
Number setae on U endopod	0	2	1	2
Number pleopods, Zoea IV, Megalopa	4	4	4	4
A 2 of Megalopa	long A 2 > P 1	short A 2 < P 1	short A 2 < P 1	medium A 2 = P 1
Yellow chromatophore over stomach	present	absent	present	absent
List of species	*bernhardus*[1] *pubescens*[1] *samuelis*[4] (?) *beringanus*[7]	*prideauxii*[1] *cuanensis*[1] *alatus*[2] *forbesii*[1,2] *marshi*[5] *annulipes*[6]	*anachoretus*[2] *kulkarnii*[3]	*longicarpus*

[1] MacDonald *et al.*, 1957.
[2] Pike and Williamson, 1960.
[3] Shenoy, 1967.
[4] Coffin, 1958, 1960.
[5] Provenzano and Rice, 1964.
[6] Thompson, 1903.
[7] Hart, 1937.

differences in detail with some additional data from Mr. Carl Nyblade, University of Washington, Seattle (personal communication).

Many differences, such as setation, might readily result from differing rates of morphogenesis in cultured versus planktonic specimens. This has been reported previously for *Pisidia longicornis* (LeRoux, 1966) and *Euceramus praelongus* (Roberts, 1968). This type of explanation is less likely for such differences as telson process 4, mandibular palp, and pigmentation. Unfortunately, no specimens of *P. longicarpus* from the field have been obtained for comparison with laboratory-reared specimens. There is little doubt, however, that Thompson was not working with *P. longicarpus*. Carl Nyblade (personal communication) has identified

Thompson's larvae as *P. annulipes* by comparison with cultured larvae of that species.

MacDonald *et al.* (1957) distinguished two groups of *Pagurus* larvae which they designated Group A (Type: *P. bernhardus*) and Group B (Type: *P. prideauxii*). Later, Pike and Williamson (1960) recognized a third group, Group C (Type: *P. anachoretus*) which is similar to Group B but differs by the absence of a median dorsal spine on somite 6, length of telson process 4 (both like Group A), and number of setae on uropod endopod (like *Anapagurus* as described by MacDonald *et al.*, 1957). Detailed comparisons of these groups and a list of the species assigned to each group to date appear in Table III. Until the present study, all described larvae of the genus *Pagurus* have fit into a previously defined group. Larvae of *P. beringanus*, described by Hart (1937), belong to Group A. Larvae of *P. samuelis*, described by Coffin (1958, 1960), appear to belong to Group A but cannot be definitely categorized because his description is not complete. Provenzano and Rice (1964) placed the larvae of *P. marshi* in Group B. Larvae of *P. kulkarnii*, described by Shenoy (1967), probably belong to Group C, accepting Shenoy's conclusion that the "normal" number of instars is 4 and not 3 as he observed. The larvae are comparable in pigmentation of the 5th and 6th abdominal somites and telson, absence of median dorsal spine on somite 6, telson process 4 long, articulated in last zoeal stage observed, and other pertinent characters except size of A 2 in the megalopa.

The larvae of Thompson (1903), herein ascribed to *P. annulipes*, were correctly placed in Group B. *P. longicarpus*, however, does not belong to this group, nor does it belong to either of the other previously defined groups. It differs from Group A by being not elongate, telson broad, having two setae on the endopod of A 2 in Zoea I and II, a curved broad antennal scale, 2 setae on the uropod endopod in Zoea IV, A 2 short and equal to right cheliped in the megalopa, no yellow chromatophore dorsal to the zoeal stomach, from Group B by possessing a long telson process 4 in Zoea III and IV and a mandibular palp in Zoea IV, and from Group C by having 2 setae on the uropod endopod and a rudimentary mandibular palp in Zoea IV. Thus *P. longicarpus* constitutes the type of a fourth group, Group D, of *Pagurus* larvae.

Additional groups of similar larvae will probably be found as further *Pagurus* species are studied. Larvae of *P. pollicaris*, based on a preliminary examination of larvae reared in this laboratory and a description of cultured larvae by another investigator (Carl Nyblade, personal communication), do not belong to any group as defined above. These larvae resemble Group B except for the lateral spines on abdominal somite 5 and the length of telson process 4 which are comparable to Group C and the A 2 scale which is comparable to Group D.

To establish whether this division of the genus based on larval characteristics is of phyletic significance, as suggested by MacDonald *et al.* (1957), will require much further study of larvae and adults with consideration of all characteristics of possible systematic value. Regardless, it has value in identification of plankton specimens.

No attempt will be made to compare larvae of the genus *Pagurus* with larvae or other genera in the family Paguridae or with larvae of the other "hermit crab" families, Diogenidae and Coenobitidae. The subject has recently been reviewed in some detail by Provenzano (1968a, b), although the emphasis was on the latter two families.

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LIFE CYCLE OF THE HYDROMEDUSA *PHIALIDIUM GREGARIUM* (A. AGASSIZ, 1862) IN THE LABORATORY

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It has been known for more than a hundred years that it is not difficult to raise hydroids from the eggs of *Phialidium hemisphericum* (Wright, 1858). There is no report of the raising of hydroids of the medusa known as *Phialidium gregarium* (A. Agassiz, in L. Agassiz, 1862) and its hydroid has not been identified until now. This would seem to be an excellent reason to undertake the observation of the whole life cycle of *P. gregarium* in the laboratory. There are, however, several equally cogent reasons. In the first place, the elaboration of a culture method which permits reliably raising hydroid colonies from single planulae, and medusae from single hydroid colonies, also enables us to study *individual* life cycles. Colonies found in nature and even colonies raised from batches of eggs are composites of many individual beings and the dynamics of their behavior are difficult to explain because the extent, the developmental stage and other specific conditions of the individual components are usually unknown. On the other hand, a hydroid "colony" derived from a single planula may be considered a single animal. By observing and comparing many of these animals, conclusions can be reached about age changes, responses to environmental conditions and about differences between animals under identical conditions which must be ascribed to genetic variability. Hydroids have been notorious for the taxonomic difficulties which they represent, and many descriptions of what are assumed to be single species show a wide and poorly delineated range of anatomical features. Detailed comparison of colonies arising from single planulae under controlled conditions can be expected to clarify some of the taxonomic issues. Finally, the breeding of medusae in clones derived from single eggs will permit more precise experimentation, for instance, on sex determination and on physiological parameters such as light sensitivity (Roosen-Runge, 1962) where results have been ambiguous until now because of genetic heterogeneity.

MATERIALS AND METHODS

All observations and experiments on living animals were made between the end of July and the end of November. Medusae were caught from the dock of the Friday Harbor Laboratories. They were brought up with a small, white enamelled saucepan on a 3½ foot handle from a float approximately 2 feet above the water. Therefore, the animals came from the surface layer down to little more than 3 feet. Freshly caught medusae, 30–40, with a preponderance of males, were left in large bowls (1000 cc) overnight and the eggs collected around 10 A.M. Special care was taken to make sure that every parent animal belonged to the species *P. gregarium* as defined by Kramp (1962).

Several settling experiments were undertaken (see Results). The simplest method proved to be the most efficient. A dozen 3 × 1 inch microscopic slides were arranged on the bottom of a large bowl containing many hundreds of planulae. After 6–12 days, 50–66% of the slides were found to have at least one primary hydroid growing on them. Such slides were hung in plastic frames, each holding 5 slides into the tanks in which most of the hydroid colonies and many medusae were raised. More than 30 cultures were observed in detail for nearly 2 months and many more were used occasionally for short-term observations.

The tanks were modified after the "Plankton-Kreisel" described by Greve (1968). The principle of the "Kreisel" is a rotary circulation in which inflow into an outer compartment and outflow from an inner compartment are separated from each other by the sand on the bottom. The "airlift" of the inner compartment was omitted as unnecessary in an open seawater system. The Kreisels (Fig. 1) were made out of carboys by removing the tops. They were 31–32 cm high and $26\frac{1}{2}$ cm in diameter. A self-syphoning outflow was arranged so that the volume of water in the tank oscillated between approximately 15 and $12\frac{1}{2}$ liters. Flow was kept between 5 and 20 liters per hour. Occasional plugging of the water lines caused irregularities and standstills which did not noticeably influence the experiments. The tanks were standing in 5–8 cm of seawater with continuous flow which assisted in maintaining a low temperature even when the circulation through the Kreisels stopped. At the inflow into the water tables, the temperature of the seawater varied from 10–13.5° C depending on the outside temperature. At an average temperature of the inflow of approximately 12.5° C, the water in the tanks had a temperature of 14° C at a flow of 14 liters per hour, 15° C at a flow of 9 liters per hour. In general, temperatures declined toward the fall.

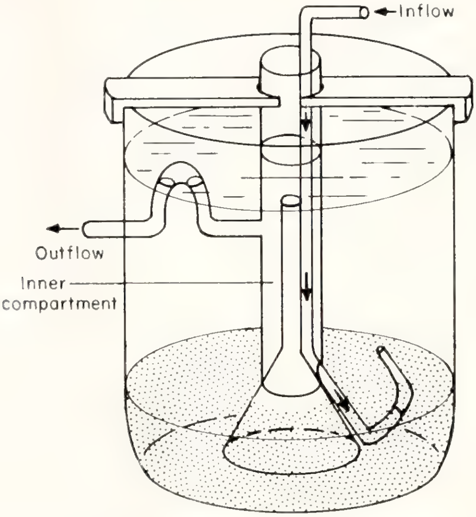


FIGURE 1. Sketch of the modified "Kreisel" used in raising hydroid colonies and medusae.

For examination and counts, the individual cultures on their slides were re-

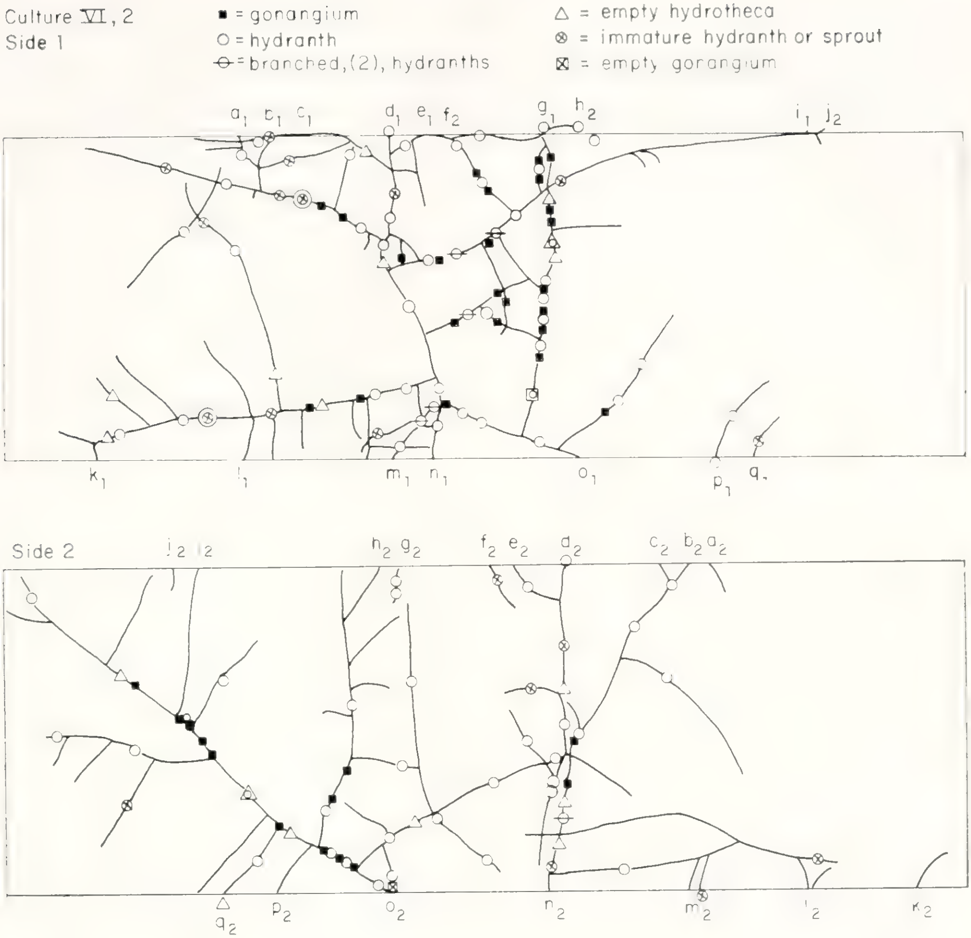

Culture VI, 2
Side 1
■ = gonangium
○ = hydranth
⊖ = branched, (2), hydranths
△ = empty hydrotheca
⊗ = immature hydranth or sprout
⊠ = empty gonangium
a1 b1 c1 d1 e1 f2 g1 h2 i1 j2
k1 l1 m1 n1 o1 p1 q1
Side 2
j2 i2 h2 g2 f2 e2 d2 c2 b2 a2
q2 p2 o2 n2 m2 l2 k2

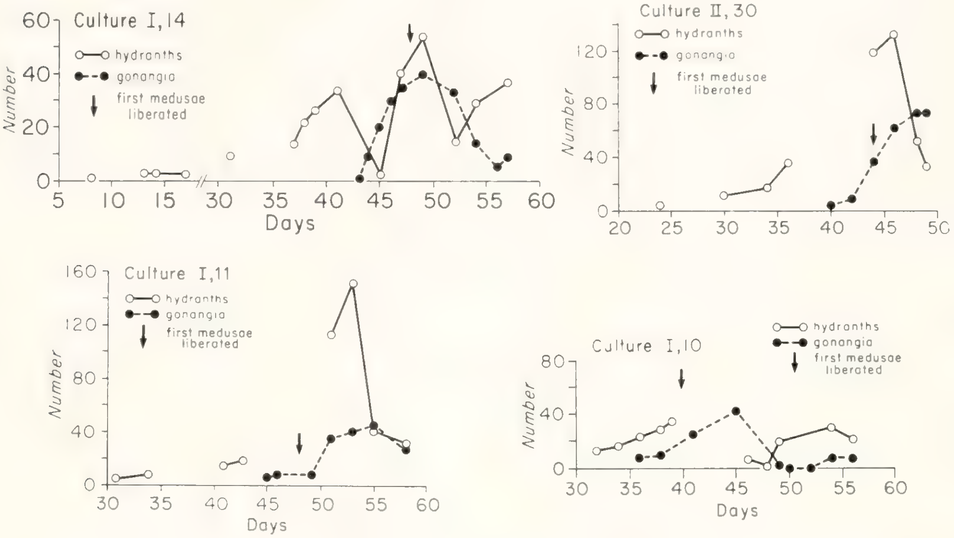
FIGURE 2a and b. Semidiagrammatic representation of a clonal hydroid culture of *Phialidium gregarium* on a 3 × 1 inch slide, after a direct tracing. The culture was drawn 34 days after the planula settled and 21 days after the first side branches appeared on the stolon. Figure 2a shows the side of the primary hydroid.

moved from the tank and inspected submersed in a Petri dish. Cultures which grew on both sides of the slides were supported by a plastic ring (diameter $\frac{3}{4}$ inch). Counts of hydroids and gonangia were made with the help of a 1 × 3 inch glass slide on which 14 areas 1.5 × 1.1 cm were drawn. This was placed directly under the culture slide and under the plastic ring.

Microscopic observations, photographs and measurements were made of living hydroids, gonangia and small medusae on a cold stage using an electric module (Cloney and Schaadt, 1970). On this stage the animals and their parts were kept very conveniently in depression slides at 10–13° C.

RESULTS

Results will be presented by first recounting observations on phases of the life cycle: settling, primary hydroids, growth of a stolon and trophosome, formation of gonosome, and growth of planktonic medusae. Secondly, the morphological features of the hydroid will be reported in detail, and the hydroid be defined summarily. This definition will then be compared with descriptions of related species in the literature, and an attempt will be made to identify the whole animal, inclusive of both its hydroid and medusal phases, by suggesting a single proper name.

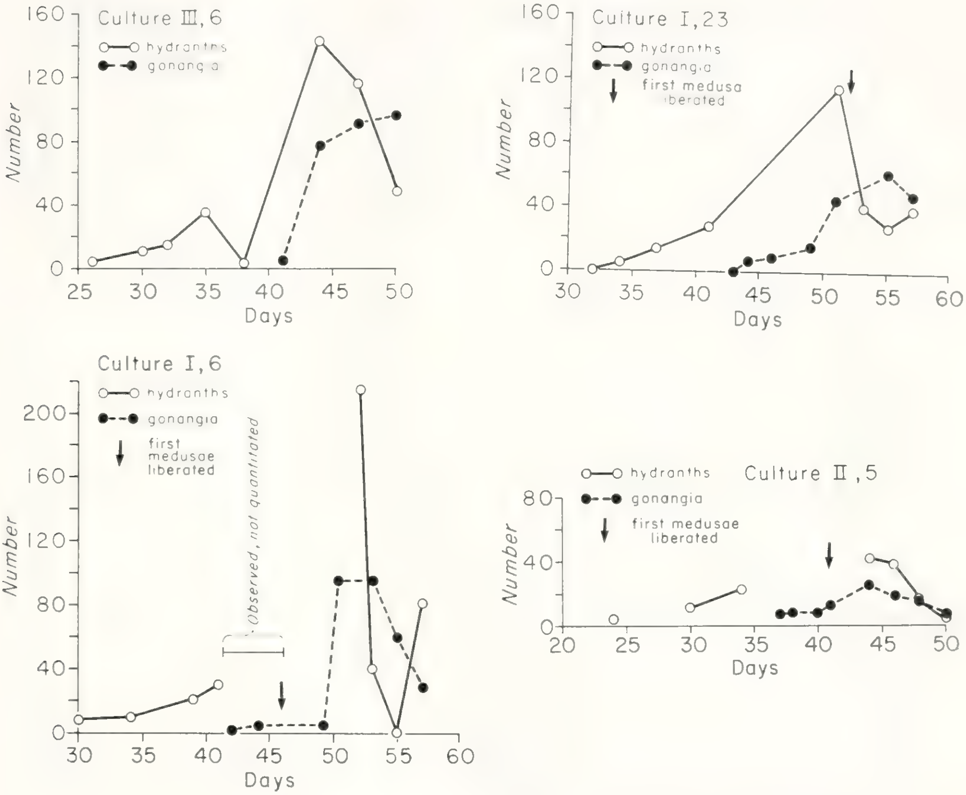


FIGURES 3–6. Graph of numbers of hydroids and gonangia during development of clonal hydroid cultures. The lines between points were drawn only when frequent observations justified the assumption of continuity of slope, even if no counts were made in the interval.

The hydroid culture—settling

The planulae of *P. gregarium* settle on many substrates with great ease; for instance, on glass, plastic, wood and algae. (The non-glycogen polysaccharide glue and the glandular cells which produce it have been discussed by Bonner, 1955.) They will settle on vertical as well as on horizontal surfaces. In one of the Kreisels in which large numbers of medusae were kept, several blank microscopic slides hanging vertically in the current began to show growth of hydroid colonies after a few weeks. In other settling experiments it was found that planulae often accumulated at the walls of the vessels near the waterline, and attempts were made to utilize this tendency by putting many slides in "staining jars" with the waterline running lengthwise along the middle of the slide. These experiments failed, perhaps, because the amount of water in these jars was very small.

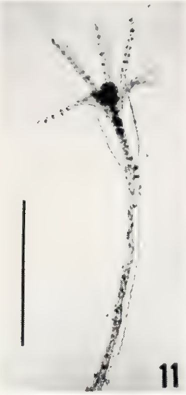
In repeated experiments with large numbers of eggs the first planulae to become sluggish and settle were observed in the second half of the third day after fertilization. Approximately half of the planulae settled on the fourth and fifth days, but even on the twelfth day a few planulae were still swimming. In this species which shows little selectivity in the substrate for its hydroid phase, the time of settlement appears to be in large part determined by an endogenous readiness of the planula.



FIGURES 7–10. Graph of numbers of hydroids and gonangia during development of clonal hydroid cultures. The lines between points were drawn only when frequent observations justified the assumption of continuity of slope, even if no counts were made in the interval.

Primary hydroids; first growth of stolon

Once a planula has settled it flattens, rounds off and spreads into a pedal disc with 4 to 6 lobes within 2 days. It then forms a stalk on which a hydranth develops. Hydranths capable of feeding usually are observed on the third or fourth day after settlement. The primary hydroid will be described in some detail below (Figs. 11–15). There appears to be a period of several days during which there is little visible change in the pedal disc. If a hydroid is fed abundantly during this period it may regress and the disc develop one or more new hydroids which are usually somewhat larger than the first.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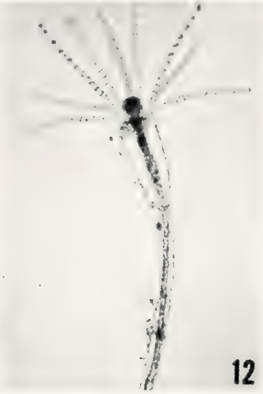
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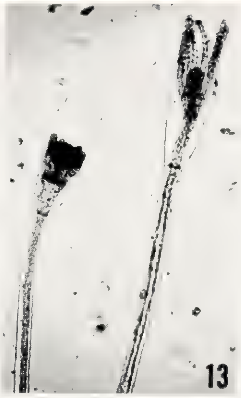
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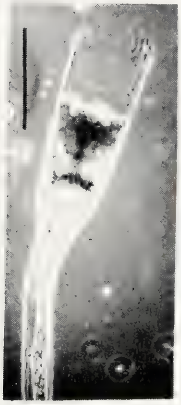

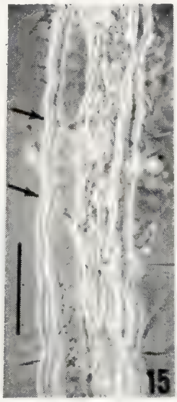
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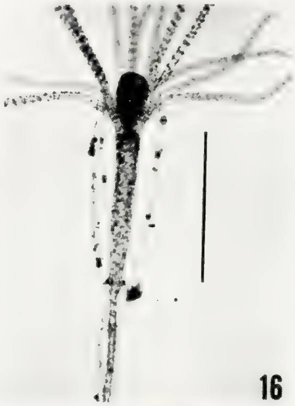
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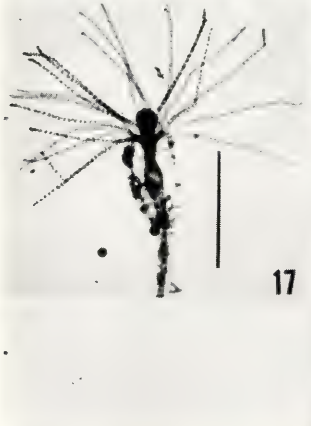
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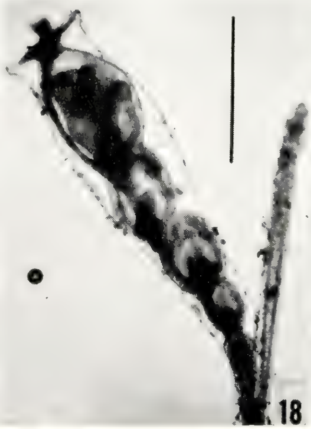
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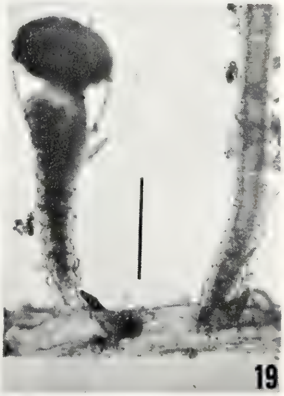
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FIGURES 11–13. Photomicrographs from living clonal cultures of *Phialidium gregarium.* (Figures 11–15 show primary hydroids.) The bar represents 500 μ (also for Figures 12 and 13.) Compare hydroids in Figures 11–13 with respect to number of tentacles, size, proportions of hydrotheca and features of annulations.

A stolon is visible usually a week after settlement. It grows frequently from one pole of the pedal disc, but sometimes from two opposite poles. Initially, the stolon advances in an almost straight line, even when it progresses in two opposite directions.

Secondary hydroids; branching of stolon

As the stolon grows, secondary hydroids are sprouting from it at rather regular intervals (1–3 mm in young cultures) which tend to become larger as the colony expands. Hydroids tend to become taller, develop more tentacles and branch more frequently (details are described below) but the degree to which this happens depends largely on the amount of food available. As long as colonies received only occasional feedings by pipette in addition to a small amount of planktonic organisms from the water supply, growth was slow and the hydroids which developed were only slightly more differentiated than primary ones. The first month of culture I, 14 (Fig. 3) serves an example. A primary hydroid was present on day 7 after fertilization. Three hydroids were counted on day 13, 14 and 17. When the culture was next examined in detail, on day 31, it had 10 hydroids. The culture had only subsisted and had, in fact, been somewhat damaged, so that the stolon was interrupted in several places. From that day on it was fed with very large numbers of brine shrimp several times daily and after 10 days there were 34 feeding hydranths, many of which were large and had 20 tentacles or more. Despite continued abundant supply of food (on day 41 the culture was completely saturated with brine shrimp under the microscope, each hydranth receiving many shrimp), within the next 4 days the number of feeding hydranths fell to 3, but in the following 4 days the number of feeding hydranths went up to 54. The eight examples in Figures 3–10 and all other cultures observed showed the same phenomenon. Boosting the food supply did always initially stimulate the growth of hydranths and, to a lesser extent, of stolon and hydroids. On the other hand, the hydranths were short-lived, if they fed heavily. A single hydranth which had ingested 6, 8 or even 10 freshly hatched brine shrimp usually regressed within 24–48 hours and only the empty hydrotheca was found in its place. Hydranths which were fed poorly often lived for many days. A lifespan of a week was observed several times. The stolon did not appear to regress after any amount of feeding. Neither did it easily respond to starvation. In cultures neglected for several weeks the stolon appeared to contain a living cenosarc

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FIGURE 14. The bar represents 200 μ. Phase optics permit identification of teeth at rim of hydrotheca. At left a "rib" may be discerned running downwards from one of the teeth.

FIGURE 15. The bar represents 50 μ. Phase picture of the distal annulations on the stem of a hydroid. Cytoplasmic processes are shown extending from cenosarc to hydrotheca in the region of a constriction.

FIGURES 16 and 17. Secondary hydroids. Bar represents 500 μ in Figure 16, 1 mm in Figure 17. Compare the hydranths in Figure 11 and 12 with that in Figure 16 (same magnification) and 17 (less than half the magnification). The hydroid in Figure 17 was 7 mm tall in life.

FIGURE 18. The bar represents 500 μ. Gonangium with 4 gonophores, arising from a stem.

FIGURE 19. The bar represents 200 μ. An immature gonangium (left), approximately 1 day old, and the base of a hydrocaulus (right) arising from a stolon. Note annulations and the difference in thickness of hydrotheca and gonotheca.

throughout, even when only a few small hydroids were present. These results are, in general, similar to those of Crowell (1953) who studied growth and regression in hydroids of *Campanularia* kept at various levels of nutrition (brine shrimp).

During the initial slow growth of the colony the stolon did not branch. The earliest branching was seen 2 weeks after fertilization in a culture which had settled 8 days previously. At first branching there were as few as 3 hydroids and as many as 10 or more. Branches tended to occur at right angles (Figs. 22–25). When branching began the culture expanded rapidly. It reached the edge of the slide somewhere before another week had passed. The edge presented no obstacle (Fig. 23). Figure 2, for example, demonstrates that 3 weeks after the first branching of the stolon a colony may have grown around the edge of the slide at 17 different points. A culture of approximately the same age not confined to a slide, but growing on the wall of the tank, extended over a circular area with a diameter of approximately 7½ cm. It appears that for the first 2 months of a colony's life the area presented by the two sides of a 3 × 1 inch slide does not limit expansion to any appreciable degree.

Growth of gonosome

The earliest formation of gonangia occurred 19 days after fertilization. This was observed several times in mass cultures from many eggs of a single spawning. In the cultures raised from one egg the earliest gonangia were seen after 21 days. In the 8 cultures represented in Figures 3–10 the first gonangia were observed on days 36–45. When a gonangium was first unmistakably recognized, it was a small, dense, elevated structure of mushroom shape (Figs. 19, 22, at arrows) very different from the thin stalks of budding hydroids. Such early stages of gonangia appeared in locations where 12 hours before there had been no indications.

Figures 3–10 demonstrate that the first appearance of gonangia is regularly preceded by an increase in the number of hydroids which begins 7–4 days earlier. For instance, culture II, 30 (Fig. 4) showed an appreciable increase in the number of hydroids on day 34, culture I, 11 (Fig. 5) on day 41 or earlier; the first gonangia were observed on day 40 and 45, respectively. Culture I, 10 (Fig. 6) demonstrates two periods of gonangial growth, the first beginning on day 36, the second on day 52. The first was preceded by a burst of hydroid formation beginning on day 32, the second by one beginning on day 48. There was no clear correlation between the peaks of hydroid development and the beginning of gonangial growth. (Compare, for instance, Fig. 7 where a peak almost coincides with the appearance of gonangia with Fig. 8 where it comes much later, or Fig. 3 where it precedes.) Nor is every rise in the number of hydroids followed by the appearance of gonangia (Figs. 3, 7).

Gonangia develop either from the stolon directly (Fig. 22) or, more rarely, from the stems of hydroids (Fig. 18). When they spring from the stolon they do so almost invariably close to a hydroid stem and characteristically equidistant on both sides of it (Fig. 22). Their location within the culture is predictable in certain respects. They occur on parts of the stolon which have reached a certain minimal age (1–2 weeks?). They never are formed by new side branches of the trophosome but are most frequently found on the middle portions of long stretches of stolon which are by their very positions identified as relatively mature. This is shown, for example, in Figure 2 which also demonstrates that the side of the slide on which the primary hydroid grew (Fig. 2a) has far more gonangia than the other side which is on the average younger. Because of the relatively short duration of the observations it cannot be stated whether there is also an upper age limit beyond which any part of the stolon becomes incapable of producing gonangia. In any case, this hydroid presents a pattern very different from one like *Podocoryne* (Braverman and Schrandt, 1969) in which a dense center is seen populated by sexual and nutritive polyps.

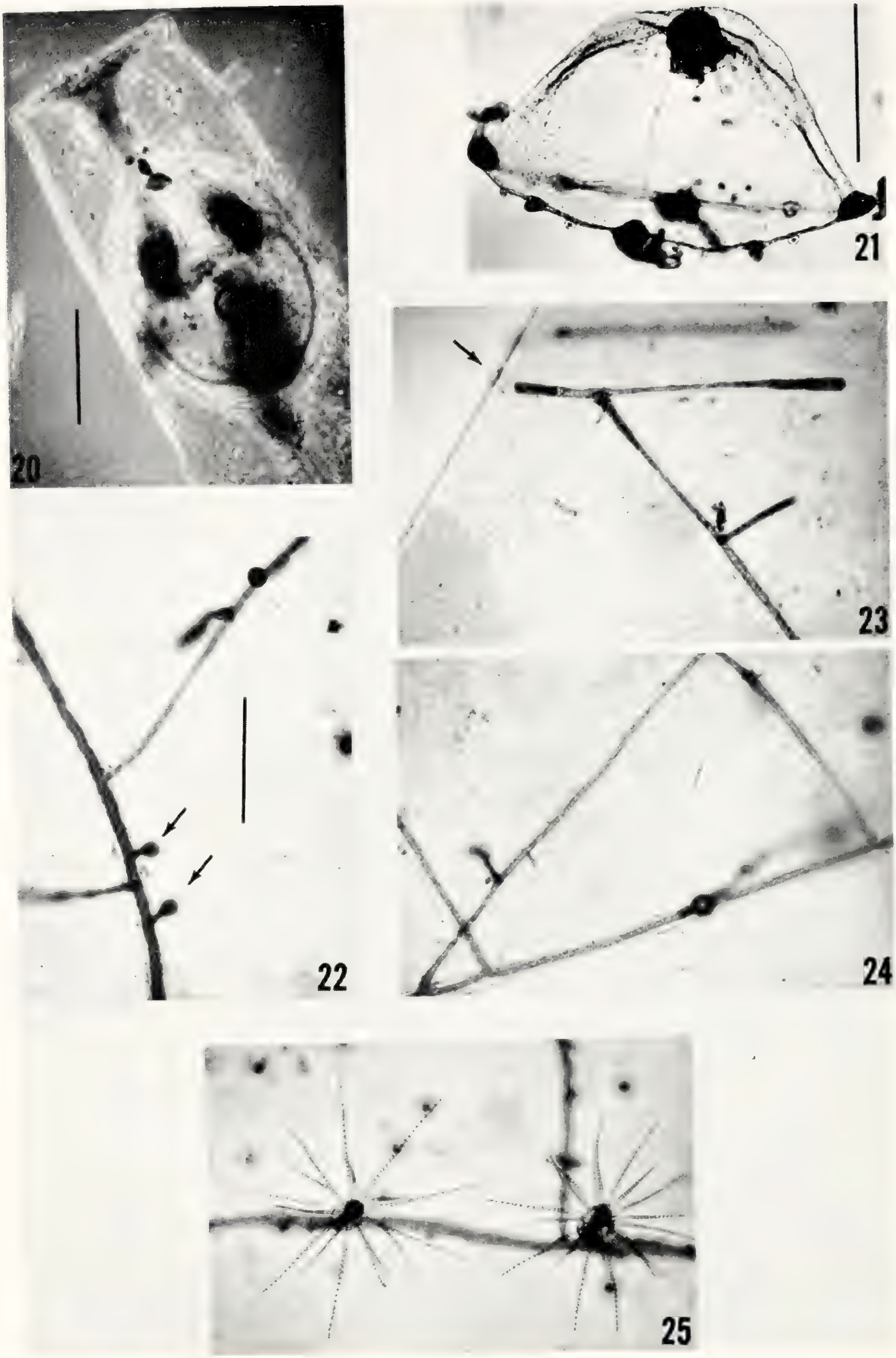
No hydroid culture was observed continuously for longer than 7 weeks. A few cultures were seen to have two phases of formation on gonangia during this period (Figs. 3, 6). The amounts of proliferation of stolon, hydroids and gonangia varied greatly from culture to culture, but appeared to be largely dependent on food supply in every case. There was no evidence that fluctuations in temperature or the lessening of daylight with approaching fall had any influence. At the end of September, 15 cultures, 2–2½ months old, were suspended from the dock at 15 foot depth for a wintering experiment. They were contained in a plastic cage which permitted open circulation. At the end of November, 6 of these cultures had been destroyed by some predator (nudibranch?). At this time, the cage was wrapped tightly in nylon screening to prevent further predation. Nine cultures were alive but much reduced from their state in September and showed only 1–6 hydranths of primary type, *i.e.*, very small with relatively few tentacles. At the end of March the cultures were once again examined. Six of them were alive and showed vigorous growth which apparently was of recent occurrence because detritus on the hydranths was minimal and empty hydrothecae were rare. The largest cultures had more than 30 hydranths with up to 20 tentacles. Three cultures showed gonangia in small numbers, in stages of development which indicated that their development had begun from 2–7 days previously.

Liberation, growth and maturation of medusae

The first medusae were liberated from hydroid cultures 26 days after fertilization. This occurred usually after 35–45 days and once after 52 days. Many cultures had not begun the formation of gonangia when observation ceased in the fall, but none of these were more than 6 weeks old, and there is no reason to assume that they would not have produced medusae eventually. All cultures observed for 52 days finally did liberate medusae. There is no indication that productivity became less with the approach of fall.

The interval between the first appearance of gonangia and the first free medusae was always between 4 and 5 days (for instance, Figs. 3–10). After medusae first appeared the cultures continued to produce for 6–10 days, which reflects the duration of a single period of formation of gonangia. During such a period 70 to more than 250 medusae were produced.

Newly hatched medusae (Fig. 21) measured 1.2–1.4 mm in largest diameter. They had 4 tentacles and 8 lithocysts, conforming in this to the defining characteristics of the genus *Clytia* (Hincks, 1868). In addition, they displayed 4 immature tentacular buds. The gonadal Anlagen were very inconspicuous but were clearly defined under higher magnifications and phase optics. The tiny medusae were able to feed on single brine shrimp while they were still connected to the blastostyle.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FIGURES 20–25.

but even when unfed they expanded rapidly to a diameter of 2.5–3 mm during the first 2 days and after that time they had 8 tentacles. Freshly hatched medusae tended to remain "hung up" within the hydroid culture, even when it was vertically suspended, and were often seen somewhat entangled in the tentacles of hydroids. There was no evidence that they were ever ingested or harmed.

A few medusae were raised to near sexual maturity in 4 weeks. These were kept in fingerbowls, were handfed and transferred into fresh seawater daily. After 3 weeks they had 23 tentacles in the average with 8 tentacular buds, and their diameter was approximately 6 mm. After 4 weeks they measured approximately 1 cm and immature oocytes were discernible in the female gonads under the microscope. It was evident that the rate of their development depended greatly on feeding, water circulation and temperature. Certainly, the conditions for their development were not optimal in the present investigation. As long as extensive attempts have not been made to establish a better method of raising medusae, possibly in the Kreisel, the fastest time of their development to sexual maturity remains unknown, but one can predict with confidence that it will be less than 4 weeks.

It is of some interest that small medusae with tentacle numbers and diameters very similar to those raised in 3–4 weeks in the laboratory were frequently found in the Friday Harbor Bay in the first 10 days of September.

Morphology of hydroids

In the following paragraphs the range of variability of features conventionally used in taxonomic identification is presented point by point for the hydroid of *P. gregarium.*

Trophosome—size and branching

A great number of primary hydroids were observed and measured. They branched only very rarely. Variations in size were relatively small. The distance from pedal disc to rim of hydrotheca was in the average 1.4 mm, the range 1.25–

FIGURE 20. Photomicrographs from living clonal cultures of *Phialidium gregarium.* The bar represents 200 μ. Phase picture of the terminal part of a 3–4 day old gonangium (same magnification as in Figure 19) showing the lid of the blastostyle and one gonophore. The upper two oval black spots are tentacular bulbs, the larger irregular black spot below is the manubrium.

FIGURE 21. The bar represents 500 μ. Medusa of *Phialidium gregarium* just after hatching. Four tentacular bulbs (2 out of focus), 4 buds of tentacles (2 out of focus) and 4 of 8 lithocysts are clearly seen. The medusa is slightly flattened in a depression slide and consequently appears wider and lower than normal.

FIGURE 22. The bar represents 1 mm in Figures 22–25. Gonangia are shown (arrows), the lower two originating on both sides of a hydroid stem departing from the stolon to the left; the upper gonangium originates near another hydroid stem seen as a hook off the stolon slightly to the left below.

FIGURE 23. A damaged stolon (arrow) winding around the edge of its slide. The cenosarc is discontinuous; the theca extends intact around the edge and the stolon is continued out of focus, above and parallel with the one on the upper side of the slide.

FIGURE 24. A crossing of one part of the stolon over another one.

FIGURE 25. The focus is on two hydranths. A characteristic pattern of hydroids and stolon branching is shown.

1.55 mm. Subsequent hydroids in an adequately fed colony are gradually increasing in size. In the beginning of colony formation, each new hydroid formed along a stolon may be larger than the last, but with the beginning of branching of the stolon new hydroids are not always bigger, but often much smaller than the last ones formed, particularly on new branches of the stolon. Branching becomes more frequent with age in most colonies, but not in all. After a few weeks some cultures show as many hydroids branching as non-branching; others may have hardly any branches but only relatively tall single hydroids. The largest single hydroid measured was 7.52 mm tall from stolon to rim of hydrotheca with tentacles 1.6–1.8 mm long. Branched hydroids occasionally exceeded a centimeter in height. As no experiments were made with a continuous maximal food supply or with different types of food, it can only be surmised that under some conditions the size of the colonies may be greater than the largest observed in the present investigation.

The variability of the hydroids of *P. gregarium* with regard to size and branching, but also with regard to number of tentacles and annulations (see below) is, perhaps, characteristic for the genus *Clytia.* Berrill (1950) has pointed out that hydroids of *johnstoni* are similarly variable in contrast to hydroids of, for instance, *Campanularia* or *Obelia.*

Tentacles. Primary hydroids (Figs. 11–14) were observed to have 8–12 tentacles, 11 in the average. The tentacles were 400 μ or less in length. Large secondary hydranths (Figs. 16, 17) had up to 25 tentacles, frequently 20–22, and the tentacles were 1.6–1.8 mm long. It should be emphasized, however, that the same colonies which possessed many large hydroids usually had some small ones with low numbers of relatively short tentacles.

Hydrotheca. The size of the hydrotheca varied greatly. The length from diaphragm to rim measured in 10 primary hydroids was in the average 440 μ, the range 385–470 μ. The diameter at the rim, usually the largest diameter, was in the average 150 μ, range 110–185 μ. In 10 large hydroids from mature colonies the average of the same measurements was 1100 μ (range 1000–1185 μ) and 410 μ (range 390–430 μ). In general, secondary hydrothecae tended to be slightly less elongate in shape than the primary ones. Hydrothecae of primary hydroids had 8–11 teeth (Fig. 14) which were sometimes pointed, but often blunt or partially broken. Empty hydrothecae often showed no teeth at all. This variability was attributed to the fragility of the thin teeth. The tentacles usually are draped over the edge of the hydrotheca within the cusps between teeth. Their movements appear to wear the rim easily, particularly when brine shrimp are fed which are large in relation to the hydranth and cause great wear and tear of the tenuous perisarc. It was difficult to find intact hydrothecae on large secondary hydroids. Usually 10 or 11 teeth were counted, but one hydrotheca possessed 13 teeth. With phase optics 4 and occasionally 8 ridges could be seen to extend downward about $\frac{1}{3}$ of the length of the hydrotheca (one is visible in Fig. 14).

Annulations. As a rule annulations were found distally, on the stem immediately under the hydrotheca (Figs. 11–14, 16) and basally where the stem sprang from the stolon (Figs. 11, 12). In primary hydroids the distal annuli varied from 3–7 (rarely more) and were in the average 33 μ high (range 28–41 μ); basal annuli varied from 12–16 and were 31 μ high (range 27–43 μ). The zones of annulation often ended sharply and the part of the stem between them was smooth (Fig. 13), but not infrequently the annuli were seen to become shallower and higher and give way to undulations throughout the center part of the stem (Fig. 11). Large secondary hydroids had a different pattern. Distal annuli were greater in number, 6–10, and much higher, 66 μ in the average (range 48–80 μ) (Fig. 16); basal annuli (Fig. 19) were less numerous, 9–14, and in the average, 48 μ high (range 35–53 μ). Cytoplasmic processes were seen which ran from cenosarc to perisarc, usually at the constrictions (Fig. 15, arrows). It was also observed that contractions of the cenosarc increased the curvature of the annuli. A thorough study of the way in which the hydrotheca is laid down and maintained may in the future bring a better understanding of the nature of the annuli, and clarify their value as a taxonomic feature. This topic has been discussed pertinently by Berrill (1950, 1961).

Gonosome

Origin, size and shape. The size and shape of a gonangium depends to a large extent on the state of its development. Small gonangia are funnel-shaped and the gonotheca is closely applied to the early gonophores. At this stage, it may appear that the pedicel is quite long and has from 5–12 annuli (Fig. 19). The pedicel is relatively shorter and has less annuli in more mature gonangia and in gonangia originating from a stem (Fig. 18). The envelope of the gonangium about the pedicel is smooth, an important distinction in comparison with the corrugated gonangium of *C. johnstoni.* A gonangium containing 5 medusae may grow taller than 3 mm and exceed $\frac{1}{2}$ mm in largest diameter which is at the equator of the largest gonophore and not at the upper rim (Fig. 20). The number of gonophores varies, however, from 2–7 and gonangia are consequently smaller or larger. Usually the majority arises directly from the stolon in the vicinity of a hydroid stem. Others arise on stems which often have 2 hydroids and may finally have also 2 gonangia. The frequency of these sites appears to vary distinctly between different colonies.

Summary definition of the hydroid of P. gregarium

Trophosome. Colonies of hydroids predominantly single but branched increasingly as a function of age and food supply. Height of primary hydroid less than 1.6 mm. Second hydroids up to 10 mm or more, depending on conditions. Hydrocaulus long, annulated at base and at top; intermediate portion generally smooth or undulating. Hydrotheca deeply campanulate, length to width 2.5–3.0:1 expanding very slightly above, with 8–13 teeth, 4 or rarely 8 fine ridges from rim downwards through upper $\frac{1}{3}$ of hydrotheca. Stolon branching predominantly at right angles.

Gonosome. Gonothecae ovate, outline smooth or somewhat undulating with a distinct collar at the opening, 3 mm or more in height when mature, arising from stolon with relatively long pedicel (up to 14 annuli) or from stem with shorter pedicel (4–8 annuli); 2–7 gonophores in each gonangium.

Taxonomic consideration

Now that the life cycle of the leptomedusa authoritatively identified (Kramp, 1962) as *Phialidium gregarium* (A. Agassiz) in L. Agassiz, 1862, page 353, *Oceania gregaria*, has been observed in the laboratory, the question of the proper scientific name of the animal must be briefly considered. This question contains two problems: (1) the identity of the hydroid which has been shown to develop from the eggs of *P. gregarium*; and (2) the selection of one name for both the planktonic and the sessile phase of the species.

Identification of hydroid. Certainly the hydroid belongs properly to the genus *Clytia* established by Lamouroux (1816) as used by Nutting (1915), Fraser (1937) and others. For convenience sake the hydroid is called *Clytia X* in the following paragraphs. Hincks (1868) in defining the genus stated as its main characteristic the production of medusae with 4 radial canals, 4 marginal tentacles and 8 lithocysts. Nutting (1915, page 53) expanded the definition by describing the trophosome: "Colony often simple but always consisting of a creeping rootstock from which spring pedicels which are not regularly branched as a rule. Hydrothecae companulate, hydranths with trumpet-shaped proboscis." All these characteristics are observed in *Clytia X*.

It is to be expected that the species of *Clytia* which liberates medusae conforming as adults to the description of *P. gregarium*, will be found among the hydroid species described for the Pacific Coast and preferably for the Northwest Pacific Coast of North America. No more than two species of *Phialidium* are ordinarily found in Puget Sound and around the San Juan Archipelago, *P. gregarium* and *P. hemisphericum*. The last is not discussed here, but I can state on good, if not conclusive, evidence, that it occurs. Fraser (1937) listed not 2 but 12 species of *Clytia* from the Pacific Coast. Five of these, namely *C. attenuata* (Calkins, 1899), *C. bakeri* (Torrey, 1904), *C. hendersoni* (Torrey, 1904), *C. minuta* (Nutting, 1901) and *C. universitatis* (Torrey, 1904), may be excluded because of their large size and completely different growth habits. *C. inconspicua* (Forbes, 1848) may be eliminated from consideration because it is in all probability synonymous with *C. johnstoni* (Alder, 1856). *C. johnstoni* has distinctive corrugations on the gonotheca. It is a well described species (Hincks, 1868; Russell, 1953) and has been clearly established as the hydroid of *Phialidium hemisphericum* (Wright, 1858). Calkins (1899) found *C. inconspicua* near Port Townsend in Puget Sound, but stated that Alder (1856) and Hincks (1868) had found it in England. Hincks reported that Wright (1858) had raised this hydroid from jellyfish, which appears to establish the fact that it is the sessile phase of *P. hemisphericum* (Wright, 1858) and not of *P. gregarium* (A. Agassiz, 1862) which has not been found in Atlantic waters.

The question remains whether any one of the three species, *C. cylindrica* (Agassiz, 1862), *C. kinkaidi* (Nutting, 1915) and *C. longitheca* (Fraser, 1914), can be singled out as uniquely fitting the description of *Clytia X*. The answer is somewhat ambiguous. *C. cylindrica* was well described by L. Agassiz (1862), page 306. In his illustration the gonangium appears very similar to that of *Clytia X*, but is described as distinctly flattened while that of *Clytia X* is round. Agassiz found this hydroid on the New England Coast where *P. gregarium* has never been observed. Fraser (1937) stated that it was found at Friday Harbor, but his description while less clear than that of Agassiz adds another discrepancy, a short pedicel with only one or two annulations. *C. kinkaidi* is found in Puget Sound (Nutting, 1915) and is similar to colonies of *Clytia X* in its early phases;

it is described as not branching and as depicted by Fraser appears to be of much smaller size than *Clytia X*. It is especially similar in the features of ribs on the hydrotheca and of long gonangial pedicels. Finally, *C. longitheca*, reported for California and Vancouver Island, is also generally similar to *Clytia X* and uniquely so with regards to its long hydrotheca. Branching is not one of its features, however, and the gonangia apparently were never observed to spring from the stems but only from the stolon.

In summary, although the above three species show many general similarities, none can be unequivocally identified with *Clytia X*. It is probable that the descriptions in the literature partly pertain to *Clytia X* in at least one of its growth phases but they remain ambiguous and confusing. It is, therefore, proposed that the hydroid of *P. gregarium* be named *Clytia gregaria*. The specific *gregarium* or *-ia*, is, indeed, the senior name of the species, if it is granted that "Clytia X" is excluded from any species previously recognized. This designation has the inherent advantage of being immediately associated with the planktonic phase of the hydroid. *C. cylindrica, kinkaidi* and *longitheca* may come to be considered synonyms of this species once the full range of variability is demonstrated.

Identification of the animal. Once an animal has been observed continuously throughout its life cycle, there is every reason to bestow a single scientific name on it. Yet, in the case of hydrozoa with two life forms, this has become a difficult matter because the traditional pattern of the nomenclature appears to present almost insuperable obstacles which can be resolved only through an inordinate amount of scholarly work. For instance, *Clytia johnstoni* and *Phialidium hemisphericum* unquestionably refer to the two phases of the same animal, but authorities, such as Russell (1953), have not proposed one proper name for the animal, and the case of *C. gregaria* and *P. gregarium* may be very similar. On first sight, the genus *Clytia* appears appropriate for reasons of priority, but there are probably difficulties in establishing the genus *Clytia* (and *Campanularia* for that matter) beyond all doubt, a task which the present author considers beyond his competence. On the other hand, one might suggest that the species discussed in the present paper be named in both its forms *Phialidium gregarium* (A. Agassiz) in L. Agassiz, 1862, p. 353. This circumvents the possible difficulty of having to revise the nomenclature with respect to the genera *Clytia* and *Campanularia* which have been thoroughly entrenched for more than a century.

DISCUSSION

It was demonstrated in the first part of this paper that the "Plankton-Kreisel" (Greve, 1968) facilitates the raising of a great number of hydroid cultures from single eggs. There is no claim that the Kreisel is the only or even the best method to do this. Rees and Russell (1937), for instance, have raised hydroids of *Amphinema, Rathkea* and *Mitocomella* in plunger jars and did not even find the renewal of water very necessary, although it proved useful for reviving unhealthy colonies. The hydroids of *Phialidium* grow quite easily in small vessels, particularly in mass cultures, if there is a continuous flow of water. Clonal cultures, however, appeared to be more visible, accessible and experimentally controllable in the Kreisels. For optimal culture conditions of small medusae the Kreisel may well be uniquely suited, but the recent investigation offered only very limited experience for medusal culture.

The availability of many individual hydroid cultures created opportunities for observation and experiment which have only been explored tentatively. Only those of most immediate interest in connection with problems of the general biology of *Phialidium* will be discussed here. There has been much speculation as to what causes the waxing and waning of swarms of jellyfish. The medusae of *P. gregarium* appear each year around the middle of April at Friday Harbor and disappear almost completely by the middle of September. Medusae of the species tentatively identified as *Phialidium hemisphericum* (Wright, 1858) behave in approximately the same way, which is of interest because this species in contrast to *P. gregarium* is circumpolar and its behavior in different localities has been well described. At Plymouth, England, large medusae are found in spring and summer (Russell, 1938, 1953), in Danish waters in winter and in spring (Kramp, 1929). On the other hand, small medusae are seen at Plymouth and in the Atlantic in midwinter and, indeed, throughout the year (Lebour, 1922). The behavior of hydroid cultures in the laboratory suggests a reasonable hypothesis on the cause of the appearance and disappearance of medusal swarms, which can be tested in the future. The hypothesis states that hydroid colonies of the genus *Phialidium* grow and produce medusae in direct response to the food supply. They react with increased formation of hydroids to major increases in the supply of brine shrimp, and a major increase in hydroid formation is followed by liberation of medusae within 2 weeks. It is evident in the laboratory that small amounts of food are not effective as stimulus; only a sustained, heavy supply of suitable food did induce medusae in the numbers necessary for the remarkably dense swarms often seen in the summer. According to the hypothesis, there should be a considerable increase in the density of planktonic organisms on which the hydroids feed 5 to 6 weeks before swarms of mature medusae are found. At Friday Harbor this appears to be the case. Johnson (1932) showed that swarms of copepods, which presumably constitute the bulk of hydroid prey showed a first noticeable rise in March and continued to increase in bursts through May. March and April also saw a great increase in barnacle nauplii. Particularly relevant may be the behavior of the copepod *Calanus finmarchicus* which appeared on limited occasions in March, April and May, very suddenly and for only a few days in tremendous numbers in the 5 years investigated. The average plankton density in surface waters usually rose in March, reached its peak in June or early July and fell precipitously in August. It is obvious that these data which take no cognizance of vertical migrations or of the preference of the hydroids for certain food organisms are not satisfactory for detailed support of the hypothesis, but they fit in a general way. A rise in plankton density in March should bring a great increase in mature medusae by the second half of April. The increase should continue until August, partly because further waves of food supply may repeatedly stimulate old hydroid colonies, and partly because the eggs of medusae spawning from April on must give rise to new hydroids and these to new medusae which will mature first in July (6–8 weeks after fertilization). It appears that the life span of individual medusae does not exceed 3 months (all findings on medusae raised in the laboratory either from hydroids or from young marine specimens support this view) so that medusae liberated at the probable peak of gonangial production in June, should disappear in September. Small growing medusae with 16–32 tentacles are, however, found until early September, but they occur infrequently and at best in small swarms of low density which must have a very low fertility.

It is obviously not a new and original finding that hydroid colonies are dependent on food supply for their growth. In fact, the results in the present paper appear to confirm, in general and in many details, the more extensive investigations of Crowell (1957) who explored the responses of growth zones in the hydroids of *Campanularia* to various nutritional levels, and who also used brine shrimp for food. He found that of all growth zones the tip of the main stolon is least affected by nutrition. Hydranth growth reacted strongly and within a very few days to restriction or increase in food. Gonangia, however, were produced only by the two best fed of 8 graded experimental groups.

From laboratory findings one would expect small numbers of medusae to be liberated even in times of relative scarcity of food because of short-term local abundance of one or the other food organism. There has been no thorough search around Friday Harbor for single medusae during the months from September to March, but occasional single specimens have indeed been found. It is important, however, to realize that in order to produce swarms of fertile density the rate of hydroid and medusal production must be extremely high, and that there is probably a critical limit below which such accumulations cannot occur. Swarms must originate in localities where production occurs with great temporal and spatial density. In laboratory cultures a few of the factors which further this can be observed. (1) The stimulus of food, provided it is of sufficient magnitude, appears to act on gonangial formation rather precisely and cause a great number of gonangia to arise within a relatively short time. (2) The liberation of medusae occurs most frequently between the hours of dusk and dawn, probably most usually in the early morning, which would tend to concentrate the release of medusae. (3) Small medusae tend to stay on the bottom and to be kept there in between the hydroids and gonangia, sharing the food supply with the hydranths. Water movement will flush large numbers of medusae out of the colonies within very short intervals of time. In addition, the medusae show a tendency to swim away at the end of the first week.

Finally, it is now possible to make an estimate within an order or two of magnitude of the productivity of a small hydromedusa such as *P. gregarium*. A female may produce an average of 50 eggs per day for 60 days (Roosen-Runge, 1962) or 3000 eggs in its lifetime. Each egg may produce minimally 300 medusae in a season. The maximal number is as yet unknown and may be many times as large. Potential productivity of each fertile couple is, therefore, at least 1 million medusae or more, which are, in the average, reduced by predators, tides and winds to another couple upholding the race.

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Roosen-Runge for technical assistance and drawings, and to Mrs. D. Ringer and Mrs. J. Tada for secretarial help.

SUMMARY

1. A method was described by which hydroid colonies, each on a microscopic slide, were raised from individual planulae of the leptomedusa known as *Phialidium gregarium*.

2. Growth and behavior of approximately 30 cultures were observed and quantitated through nearly 2 months. Cultures produced gonangia and medusae 3–7 weeks after fertilization of the egg.

3. Gonosome development was always preceded by a burst of hydroid development 5–9 days previously. This burst appeared to be initiated entirely by an abundant food supply (brine shrimp).

4. Medusae were liberated 4–5 days after gonangia first appeared. From the growth rate of medusae under the less than optimal laboratory conditions, it was estimated that sexual maturity may be reached in approximately 3 weeks. The lifespan of the medusae probably does not exceed 3 months.

5. A small number of cultures wintered in an open cage in the sea. At the end of November they were found in a greatly reduced state and possessed only a very few, very small hydranths. At the end of March they were in the early phases of vigorous trophosomal growth and the gonosome had just begun to flourish.

6. From the behavior of clonal colonies in the laboratory, the hypothesis was derived that the swarms of mature medusae in nature are the direct result of a "bloom" in zooplankton which occurred 5–7 weeks previously and stimulated first hydroid development and in consequence the formation of gonangia.

7. Morphological characteristics of the hydroid colonies were described and their variability under laboratory conditions recorded and discussed. It was shown that many taxonomic features such as size, branching, number of tentacles and annulations depend quantitatively on the age of the individual colony, its state of nutrition and on genetic factors.

8. The hydroid belongs to the genus *Clytia* (Lamouroux, 1816). A careful comparison was made with species of the genus as described for the Puget Sound region and California. It was concluded that none of these delineates unambiguously the morphological features of this hydroid. A tentative name for the hydroid was assigned according to priority principles as *Clytia gregaria*. It was suggested that *Phialidium gregarium* may be the most appropriate name for the species in both its phases.

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NATURAL AND SYNTHETIC MATERIALS WITH INSECT HORMONE ACTIVITY. 5. SPECIFIC JUVENILE HORMONE EFFECTS IN ALIPHATIC SESQUITERPENES

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Insects belonging to different taxonomical groups often show great differences in their sensitivities to juvenile hormone analogues. For example, some compounds, such as the esters of monocyclic sesquiterpenes, have substantial activity on the hemipteran insects of the family Pyrrhocoridae, but little or no activity for most other insects including other families of the Hemiptera (Sláma, Suchý and Šorm, 1968). By contrast, there are many compounds, such as methylenedioxyphenylethers of geraniol (Bowers, 1969), which show great activity on pupae of Coleoptera but only slight activity on hemipterans.

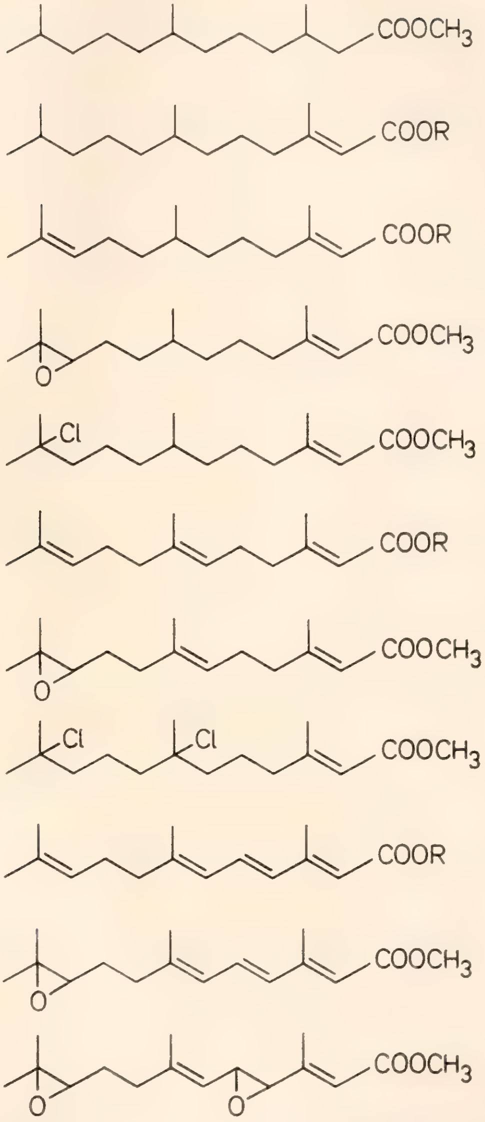
In previous studies of juvenile hormone activity of farnesenic acid esters (Sláma, Romaňuk and Šorm, 1969) we have noticed that minor changes in chemical structure, such as the introduction of hydrogen chloride across the 6,7 and 10,11 double bonds, may lead to as much as 10,000-fold increase in activity for the bug *Pyrrhocoris apterus* with a simultaneous 100-fold loss of activity for the beetle *Tenebrio molitor*. Other studies on hemipteran insects (Suchý, Sláma and Šorm, 1968) have documented the considerable differences between the families or higher taxonomic groups in their response to individual juvenile hormone analogues.

In the present investigation we have explored the phenomenon in further detail. To this end we have determined the juvenile hormone activity of each of a series of fifteen synthetic esters of aliphatic sesquiterpenic acids when tested on eight genera of insects including one or more representatives of three families of Hemiptera and two families of Coleoptera. Special attention was centered on those features of molecular structure which enhance or detract from hormonal activity for individual families and genera of insects.

MATERIALS AND METHODS

Juvenile hormone activity was assayed on the following Hemiptera: *Pyrrhocoris apterus* L. and *Dysdercus* (*Paradysdercus*) *cingulatus* (Fabr.) (family Pyrrhocoridae); *Lygaeus equestris* L. (family Lygaeidae); *Graphosoma italicum* Müll., *Aelia acuminata* L., and *Eurygaster integriceps* Put. (family Pentatomidae). Each analogue was also assayed on the following Coleoptera: *Tenebrio molitor* L. (family Tenebrionidae) and *Dermestes vulpinus* Fabr. (family Dermestidae).

For topical assays the compounds were applied in a standard 1 μl drop of acetone on uninjured cuticle of freshly molted last instar larvae (Hemiptera) or freshly molted pupae (0–20 hrs) (Coleoptera). For injection assays on *Tenebrio*


COOCH$_3$
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COOCH$_3$
O
O

I

II $R = CH_3$
III $R = C_2H_5$

IV $R = CH_3$
V $R = C_2H_5$

VI

VII

VIII $R = CH_3$
IX $R = C_2H_5$

X

XI

XII $R = CH_3$
XIII $R = C_2H_5$

XIV

XV

FIGURE 1. List of the compounds used for assays of juvenile hormone activity.

and *Dermestes* the compounds were injected in a 1 μl drop of olive oil into the body cavity of freshly molted pupae. The activity was determined according to the degree of retention of the larval (Hemiptera) or pupal (Coleoptera) characters after the next ecdysis.

The dose-response experiments performed on *Pyrrhocoris* and *Graphosoma* revealed that the whole range of activity from zero (formation of perfect normal adults) to maximum (formation of morphologically perfect supernumerary larval instars) was realized with a 10-fold change in concentration. The reciprocal increase of larval epidermal patterns and decrease of adult patterns was linear when plotted against the logarithmic scale of concentrations. This allowed determination of a standard intermediate effect with minimum error. In the pupae of Coleoptera the range of activity from zero to maximum (extra-pupal instars) was realized with 100 to 1000-fold concentration change. The activity is given in "ID-50 Morph." units. This unit indicates the amount of substance in micrograms per specimen which produces under the above described conditions of application half-larval (Hemiptera) or half-pupal (Coleoptera) adultoids. The unit concentration occurs in the middle of the concentration range necessary for zero to maximum effect. The concentrations provoking the first signs of activity can easily be derived from the ID-50 values. For example, if the ID-50 for a hemipteran larva is 0.05, the first signs of activity occur at 0.01; so also, maximum activity would be attained by the application of approximately 0.1 μg. Each value in Table I represents a result of 4 to 5 tests at different concentrations, each concentration being assayed on 5 to 10 individuals.

The list of the compounds is presented in Figure 1. The compounds II to V, VIII, XII, and XIII were prepared by means of Wittig's reaction in which the aliphatic methyl ketones were treated with carbomethoxymethylene-triphenylphosphorane (for preparation of methyl esters) or carboethoxymethylene-triphenylphosphorane (for preparation of ethyl esters). This method yielded rather pure products.

The starting material for preparation of compounds II and III was hexahydropseudoionone, for IV and V citronellylacetone, for VIII geranylacetone, and for XII and XIII pseudoionone. Compound I was prepared by hydrogenation of VIII in presence of Pd/C catalyst. Compound VII was obtained from IV by addition of hydrogen chloride. The epoxides VI, X, and XIV were obtained from IV, VIII and XII after treatment with perphthalic acid. Similar technique was used to prepare the diepoxyderivative XV (two equivalents of perphthalic acid were used). Compounds IX and XI were kindly provided by Dr. M. Romaňuk.

All the synthesized compounds were purified by thin-layer or column chromatography on silica gel and their purity was checked by gas-liquid chromatography (with the exception of halogen-containing compounds). In some cases infra-red and mass spectrometry was used for further characterization.

RESULTS

All the compounds studied were methyl or ethyl esters of aliphatic C_{15} terpenoid acids which differed in the amounts and positions of the double bonds. For easier orientation we have divided the compounds into the following groups:

Group **A**—Methyl ester of 3,7,11-trimethyl-dodecanoic acid (I) with fully saturated molecule.

B—Esters of 3,7,11-trimethyl-2-dodecenic acid (II, III) with one double bond.

C—Esters of 3,7,11-trimethyl-2,10-dodecadienoic acid (IV, V) with 10,11-epoxy (VI) and 11-chloro (VII) derivatives.

D—Esters of 3,7,11-trimethyl-2,6,10-dodecatrienoic acid (VIII, IX) with three double bonds including also 10,11-epoxy (X) and 7,11-dichloro (XI) derivatives.

E—Esters of 3,7,11-trimethyl-2,4,6,10-dodecatetraenoic acid (XII, XIII) with four double bonds including 10,11-epoxy (XIV) and 4,5,10,11-diepoxy derivatives (XV).

The juvenile hormone activity of simple esters

As summarized in Table I, the saturated ester (I) showed no detectable activity except when high concentrations were administered to pyrrhocorid bugs. By contrast, substantial activity was recorded for the esters (II, III) of group B which contain the conjugated double bond at C_2. These compounds showed a 10- to 20-fold increase in activity for the pyrrhocorids, and the appearance of low but definite activity for the pentatomids. In the case of the two species of Coleoptera, the increase in activity was at least a thousandfold.

TABLE I

Juvenile hormone activity of the compounds listed in Figure 1. The values indicate ID-50 (Morph) units in μg per specimen

Family	Pyrrhocoridae		Lygaeidae	Pentatomidae			Tenebrionidae		Dermestidae	
Species	*Pyrrhocoris apterus*	*Dysdercus cingulatus*	*Lygaeus equestris*	*Graphosoma italicum*	*Aelia acuminata*	*Eurygaster integriceps*	*Tenebrio molitor*		*Dermestes vulpinus*	
Application	topical	topical	topical	topical	topical	topical	injection	topical	injection	topical
Compound No.										
I	100	100	>500	>1000	>1000	>1000	>1000	>1000	>1000	>1000
II	5	10	7	100	70	>100	1	10	1	10
III	5	10	5	50	50	—	1	7	0.5	5
IV	5	5	3	50	50	100	5	20	5	—
V	5	5	3	8	—	—	5	10	5	10
VI	0.5	0.07	0.5	1	4	—	3	10	3	9
VII	0.08	0.05	0.9	5	5	—	1	1	5	5
VIII	50	50	50	50	50	100	10	100	8	—
IX	50	30	40	50	30	100	5	100	5	—
X	3	1	—	10	5	20	10	—	—	—
XI	0.0008	0.005	0.009	30	1	30	1000	>1000	25	50
XII	3	1	5	3	5	20	500	>1000	100	—
XIII	3	1	—	1	5	20	500	>1000	100	—
XIV	0.8	0.7	1	1	7	5	100	>1000	—	—
XV	0.8	0.7	1	5	7	8	>1000	>1000	>500	—

The tested compounds contained approximately $\frac{2}{3}$ *trans* methyl-3 isomers.

With further increase in the number of double bonds the activity undergoes definite changes distinctive of each taxonomic group. The results may be summarized as follows: (i) In the Pyrrhocoridae and Lygaeidae the activity does not show any considerable variations with increasing degree of unsaturation, *i.e.*, critical doses are between 1 and 10 μg with the exception of farnesenic acid esters (VIII, IX) which are less active. (ii) The Pentatomidae show a continuous increase of juvenile activity with increasing degree of unsaturation; the difference is approximately 10 to 100-fold. (iii) Just the reverse is true for the coleopterans, *Tenebrio* and *Dermestes*, where the esters of group B (II, III) are the most active and the highly unsaturated compounds of group E (XII, XIII) are 100 to 1000 times less active or completely inactive.

The effect of epoxidation

When the 10,11 double bond of the compounds IV and V is saturated by an oxirane ring (VI), hormonal activity is substantially increased in hemipteran insects and slightly increased also in the beetles. The increase is smaller in XIV where the oxirane ring is placed across the 10,11 double bond of the highly unsaturated compound XII. The diepoxy-ester (XV) with both 10,11 and 4,5 double bonds epoxidated has approximately the same activity as the monoepoxy-ester (XIV), suggesting that the oxirane ring in the position 4,5 does not increase the activity as that in the position 10,11.

The effect of hydrochlorination

Substitution of the 10,11 double bond in compounds IV and V by hydrogen chloride has similar effects on hormonal activity as described above for the corresponding epoxy-derivative. When both the 10,11 and 6,7 double bonds of compounds VIII and IX are saturated by hydrogen chloride (XI) there occurs an enormous increase in activity for pyrrhocorid and lygaeid bugs, whereas little change in activity is observed for pentatomids. Equally impressive is the great decline in activity for the beetles. Since the monohydrochlorinated compound VII is highly active on beetles, the 6,7 double bond and especially the status of C_7 seem crucial. Thus, when the C_7 is attached to a halogen atom or is bound to an oxygen atom of an epoxide, the compounds are very active on pyrrhocorids and very inactive on the beetles. By contrast, in the pentatomid bugs the activity is little influenced by changes at C_7.

TABLE II

Summary of the relationships between the double bonds, their substitutions by hydrogen chloride and epoxide, and juvenile hormone activity

	Pyrrhocoridae Lygaeidae	Pentatomidae	Tenebrionidae Dermestidae
Increasing degree of unsaturation	little or no change	increase	decrease
10, 11 substitution	large increase	increase	slight increase
6,7 substitution	large increase	slight decrease	large decrease
6,7 and 10,11 substitution	enormous increase	slight increase	large decrease

The effect of stereochemical isomers

The *cis* and *trans* isomers of compounds II and VII were isolated and tested on *Pyrrhocoris*, *Dysdercus*, and *Graphosoma* by topical assays and on *Tenebrio* by both topical and inject assays. The *trans* isomers were found approximately 10 times more active than the *cis* isomers in all the hemipterans. In *Tenebrio* there were large variations in activity but, in general, the *trans* isomers appeared to be 5 to 100 times more active than the *cis* isomers.

The effect of methyl and ethyl ester radicals

In the present study we found rather small difference in the activity of methyl and ethyl esters on pyrrhocorids, and relatively small activity increase of ethyl esters over the corresponding methyl esters in other insects studied. Present indications are that the ethyl esters cause a greater increase in activity in compounds where the 10,11 double bond is saturated by hydrogen chloride or epoxide.

DISCUSSION

Jarolím, Hejno, Sehnal and Šorm (1969) have described the juvenile hormone activity of the compounds listed in Figure 1 on larvae and pupae of *Galleria mellonella* (Lepidoptera, family Pyralidae). It appears that the action of these compounds on *Galleria* is in many respects similar to that which we have found on pupae of the beetles, *Tenebrio* and *Dermestes.*

It is known from the literature that there are certain structural features of juvenile hormone analogues which produce a general increase or decrease of activity in all species. Our results have confirmed these generalizations which can be summarized as follows: (i) higher activity of stereochemical *trans* isomers (Yamamoto and Jacobson, 1962; Röller and Dahm, 1969; Wigglesworth, 1969a; Sláma *et al.*, 1969; (ii) necessity of the 2,3 double bond in aliphatic terpenes; (iii) loss of activity after introduction of very polar groups such as hydroxyls (Suchý *et al.*, 1968); (iv) increased activity after addition of 10,11-epoxy group (Bowers, Thompson and Uebel, 1965; Röller and Dahm, 1969; Ratuský, Sláma and Šorm, 1969; Wigglesworth, 1969a, 1969b; Jarolím *et al.*, 1969); (v) increased activity after additions of hydrogen chloride at the 10,11 double bond (Law, Yuan and Williams, 1966; Romaňuk, Sláma and Šorm, 1967; Sláma *et al.*, 1969; Wigglesworth, 1969a); (vi) higher activity of ethyl esters (Law *et al.*, 1966; Röller and Dahm, 1969; Sláma *et al.*, 1969; Wigglesworth, 1969a).

As indicated in the present study, there are certain chemical changes which lead to predictable increase or decrease of the juvenile activity for individual families and orders of insects. These have been summarized in Table II. The structure-activity relationships of this type are not common in the literature for they require large biological screenings on representatives of several insect groups. We have already mentioned that the degree of unsaturation and the chemical configuration at C_7 are among the factors determining selective action in esters of aliphatic sesquiterpenes. Both these factors seem to be associated with one and the same biological mechanism since each group of insects which shows specific responses to the degree of unsaturation is also sensitive to substitutions at C_7.

We suspect that this type of information will be helpful in the preparation of new synthetic juvenile hormone analogues with more or less selective pesticide effects.

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SUMMARY

The juvenile hormone activity of ethyl or methyl esters of aliphatic sesquiterpenic acids with 0 to 4 double bonds was tested on 8 species of insects belonging to 5 families of Hemiptera and Coleoptera. Special attention was paid to the addition of hydrogen chloride or epoxide groups on or across the double bonds.

Certain chemical changes in the molecule appear to cause a general increase of the activity in all species studied. These are: the presence of 2,3 unsaturation conjugated with the carboxyl group; the *trans* stereochemical position of the C-3 methyl; an introduction of 10,11 epoxide or hydrochloride; and esterification with ethyl rather than with methyl.

There are also chemical changes which lead to genus- or family-specific variations in juvenile hormone activity. With increasing amount of unsaturation the activity either remains almost unaffected (pyrrhocorid bugs) or increases (pentatomid bugs) or decreases considerably (tenebrionid and dermestid beetles). The addition of hydrogen chloride or epoxide to the 6,7 double bond causes enormous increase in the activity in the Pyrrhocoridae and Lygaeidae, no considerable change in the Pentatomidae, and great decreases in the beetles and Lepidoptera.

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EFFECTS OF SUPERABUNDANT OXYGEN ON THERMAL TOLERANCE OF GOLDFISH

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The physiology and ecology of thermal tolerance in teleosts has long been the subject of research, and several extensive articles and reviews on this topic have appeared in recent years (Brett, 1956; Fry, 1958, 1967; Fisher, 1958; Weatherley, 1963a, b). Temperature tolerance is greatly affected by physiological processes, which are governed by the normally prevailing temperatures of the environment, and there has therefore been much study of the relationship between lethal temperature and acclimation temperature (*e.g.*, Brett, 1956; Fry, 1967). The fundamental biological characteristics of impairment of animal function through high temperature have, however, remained rather obscure (see Fry, 1967). Nor is there yet much understanding of the effects of environmental factors, other than acclimation temperature, on thermal tolerance. The importance of oxygen tension, salinity level, pH, *etc.*, are, for instance, largely unknown.

Hypothesis of Oxygen-Dependent Thermal Tolerance

Despite the lack of real understanding mentioned above, it has, at least, become evident that simplistic ideas of the overriding importance of some single cause of heat death are unlikely to be satisfactory (Rose, 1967). On the other hand, it does appear likely that certain aspects of the physiology of the whole animal will prove more directly sensitive to high temperature than others (Weatherley, 1963a). Thus, it has been thought for many years that functional failure of the nervous system may be of key importance (Brett, 1956; Fisher, 1958; Fry, 1958, 1967). This suggestion has considerable logical appeal in view of the long-known vulnerability of vertebrate nervous systems to even short-term deprivations of oxygen. We might, for instance, advance an argument along the following lines to account for impairment of the animal through damage to the nervous system when exposed to high temperature.

If ambient temperature rises substantially, then oxygen requirements of all tissues may be expected to show a corresponding increase. However, the circulatory system would be able to meet the initial need for an increase in blood flow, and so supply the additional oxygen required. Should their demand begin to exceed the maximum ability of the respiratory and circulatory systems to supply oxygen, some tissues may be expected to begin to suffer from an oxygen shortage. The severity and permanence of their resulting malfunction will then depend on their particular sensitivity to that shortage. Nervous tissue will be among those tissues—with a high inherent metabolic rate and marked sensitivity to oxygen lack—most rapidly and seriously affected. As the vertebrate respiratory/circulatory system is, at least partly, under the control of the nervous system, we can expect the establishment of a 'vicious cycle' of worsening failure of both systems, if the oxygen shortage is maintained. In addition, the systems for oxygen transport and for oxygen and carbon dioxide exchange in vertebrates may, of course, be directly affected by temperature.

An experimental finding for trout and roach by Alabaster and Welcomme (1962) lends support to these ideas. They found that if the oxygen tension in the water is significantly below air-saturation value, thermal tolerance is appreciably lowered.

The present paper describes experiments performed to test the hypothesis that the lethal temperature of goldfish may be affected by failure of the oxygen supply system. The method used was to augment the normal oxygen supply, in the expectation that a superabundance of oxygen might ensure ready entry of oxygen into the fish through the entire body surface. Thus, a functional failure of the respiratory, circulatory, or oxygen transport systems would be compensated, at least to some extent, by saturation of blood serum, lymph, tissue fluids, etc., with oxygen.

MATERIALS AND METHODS

Plan of experiments

A first testing of the hypothesis was to compare mean survival time at a fixed upper lethal temperature among fish in oxygen-enriched water ('experimentals') with those in water at air-saturation ('controls'). The oxygen available to the 'controls' was limited to the quantity that dissolves in water at the altitude of Canberra (approx. 600 m). In this first group of experiments all goldfish were removed directly from an aquarium stock tank and placed immediately either into the oxygen-rich water or ordinary 'air-saturated' water in the experimental vessels.

In the second group of experiments batches of fish were placed in oxygen-rich water for periods of 2, 6¼ and 27½ h before testing them against 'controls', or normal 'experimentals' (those placed in oxygen-rich water immediately before testing).

The results of these two groups of experiments (Tables I and II), indicated the desirability of determining the ability of very much higher concentrations of oxygen to ameliorate thermal stress. A third series of experiments was therefore carried out in a specially constructed compression chamber (Fig. 1), and the results are given in Figure 2.

The fish

The goldfish *Carassius auratus* used in these experiments, which were obtained from a commercial supplier, were mostly of the type known as 'comets', except for a few experiments (1 and 2, Table I) in which 'calicoes' were used. In any one series of experiments the fish were of relatively uniform size and were sexually immature. The fish were maintained in aerated, filtered water in glass aquarium tanks and received 12 hours of light and 12 of darkness per day. They received a standard fish food every two days, similar to that described by Weatherley (1963a), and for which they showed good appetite.

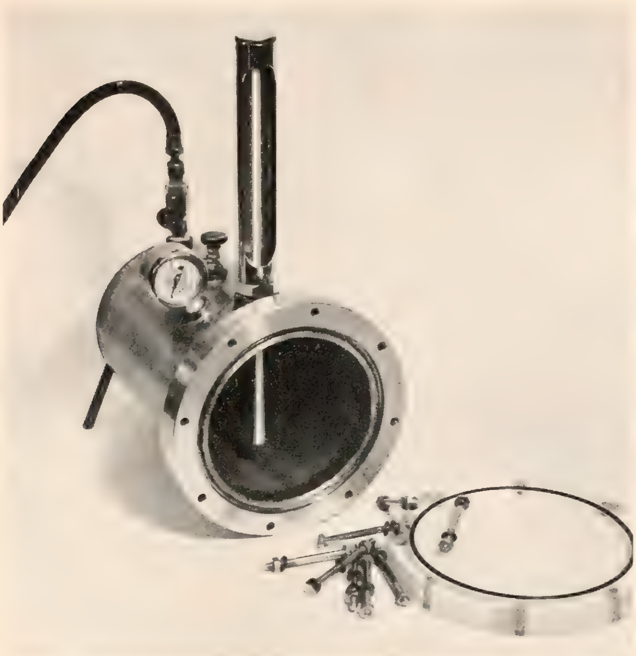

FIGURE 1. Compressor unit (4.4 l capacity) for experiments on upper lethal temperature at high tensions of oxygen. Chamber is constructed of stainless steel, with perspex end plate. Thermometer, pressure gauge, bleeder valve and high pressure oxygen hose are shown.

TABLE I

Survival times of goldfish in superoxygenated water compared with controls in air-saturated water when exposed to lethal temperature of 40°C. (approximate acclimation temperature 28° C).

Experiment No.	Treatment	No. of individuals n	Mean survival time (sec) $\bar{x}$	Sums of squares Σd^2	Standard deviation of diff. of means σd	't'	Significance level (%)
1	Control	8	192	64397	50.5	19.6	0.1
	High oxygen	8	1181	1245688			
2	Control	8	264	206988	67.3	10.8	0.1
	High oxygen	8	989	2117888			
3	Control	8	528	957552	80.1	8.0	0.1
	High oxygen	8	1170	2330400			
4	Control	6	587	323334	75.7	7.8	0.1
	High oxygen	6	1175	913350			
5	Control	8	203	152152	45.1	12.0	0.1
	High oxygen	8	746	890588			

In general, acclimation temperatures in the stock tanks varied by ± 0.5° C daily about the means as indicated below and in Tables I and II; Fig. 2. In any one series of experiments the fish were either drawn randomly from one large stock tank in which all had spent at least a fortnight prior to experiments, or from several smaller adjacent tanks with identical temperature records. Sample bias is believed to have been kept to a minimum by these procedures.

Oxygen-supersaturated water at normal atmospheric pressure.

For the first series of experiments the test containers consisted of two large beakers, each containing approximately 2 liters of aquarium water (Table I). In the second series of experiments (Table II) the somewhat larger size of the fish required the use of even larger beakers of 5 liters capacity. In both series of tests a temperature was selected as 'lethal' on the basis of simple preliminary pilot tests combined with consideration of the published records of lethal temperatures for goldfish at various acclimation temperatures (Brett, 1956). With these guides as a basis, 40° C proved convenient in experiments 1–5 (Table I), whereas in experiments 6–10 (Table (II), because of differences in the size and age of the fish and their acclimation temperature, 35° C was used. Temperature was maintained in each beaker at the selected lethal value by placing them in a glass tank in which the water was kept at a constant temperature by a Braun 'Thermomix'. Temperatures in the beakers varied from the selected lethal temperature by no more than ± 0.05° C; in most instances no changes at all were detected.

TABLE II

Analysis of survival of goldfish held in oxygen-saturated water for various times before exposure to a lethal temperature of 35° C, compared with controls in air-saturated water (approximate acclimation temperature 18° C).

Experiment No.	Treatment	No. of individuals	Range of survival times (sec)	Sums of E scores preceding each C score (Mann-Whitney 'U' test)	Significance level (%)
6	Control	8	70–555	—	—
7	Placed in O_2-sat. water immediately before test	8	195–>7200	4	0.1
8	2 hr in O_2-sat. water before test	8	160–>7200	14	3.2
9	6¼ hr in O_2-sat. water before test	8	295–>7200	3	0.1
10	27½ hr in O_2-sat. water before test	8	180–>7200	12	1.9

C = 'control' values for survival times; E = 'experimental' values for survival times.

In the 'control' beakers the water was aerated, with air at 3 lb per sq inch pressure passing through an aquarium airstone, for at least 10 min before each experiment. In the 'experimental' beakers water was supersaturated with oxygen by vigorous passage of pure oxygen for about 10 minutes.

Determinations of oxygen concentration in both containers at the atmospheric pressure of Canberra and the acclimation tempertures of these experiments (17–28° C) showed the saturation value to be 92–93% of the sea level value, and 4–5 times this as a result of superoxygenation. The oxygen tension in the 'experimental' beakers was thus always beyond the range of an oxygen electrode, and was therefore determined by the Winkler method, using 100 ml samples.

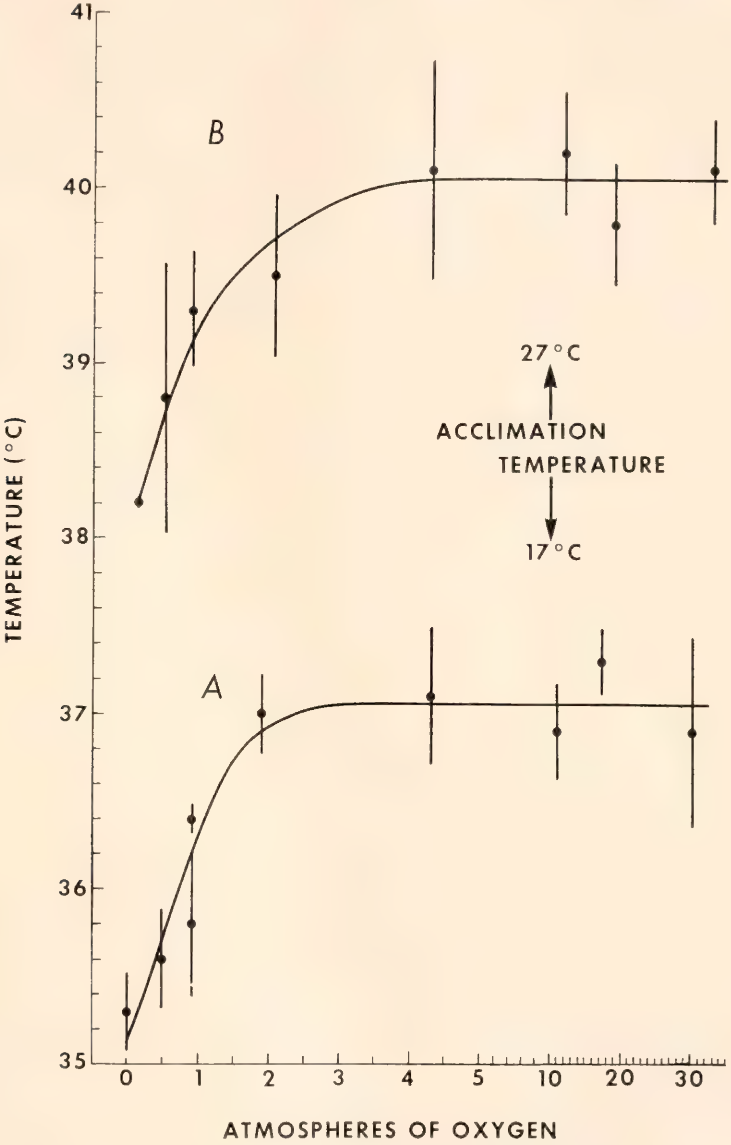

FIGURE 2. Effects of increasing concentrations of dissolved oxygen on mean upper lethal temperature (black circles), based on samples of five fish per point. Lower curve (curve A in text) was for fish acclimated at 17° C, upper curve (B in text) for fish acclimated at 27° C. Vertical bars represent twice the standard deviation of each mean. See text for further explanation.

In the beakers, the water was continuously aerated ('controls') or oxygenated ('experimentals') during the actual exposures of fish to lethal temperatures.

The method of testing was to expose samples, usually of 8, occasionally of 6 or 7, fish to the test temperatures by placing all fish simultaneously into the beakers. Times to death were recorded for each individual. The criterion of death was cessation of movement, which was sometimes rather difficult to observe, though judgment rapidly improved during a few pilot tests. Cessation of opercular movement appeared to be closely correlated with that of other movements of the body, so it was frequently employed as a convenient and sensitive index of death. Many fish judged 'dead' by the criterion of 'cessation of movement' could be readily revived if removed immediately to a cold surface or cool water, but, by the same token, fish in which movement had ceased failed to move again if maintained at the same temperature or if the temperature was raised still higher.

On removal from the lethal temperature test beakers fish were weighed and measured. pH in the beakers was measured before and after each lethal test.

Higher concentrations of oxygen

The results of exposing goldfish to concentrations of oxygen higher than those obtainable by vigorous aeration alone (Tables I and II) made it desirable to examine the effects of much higher tensions of oxygen. The construction of a compression chamber was needed for this investigation (Fig. 1). The chamber was built of $\frac{1}{8}$ inch seamwelded stainless steel, in the form of a cylinder. A 1-inch-thick perspex end wall may be bolted to a heavy steel collar welded to one end; an airtight seal is effected by means of a rubber O ring. The unit carries a pressure gauge, a tap from which rubber pressure hose leads to an oxygen cylinder, and a steel-sheathed thermometer which dips into the water of the chamber when in use.

Before use, the compressor was placed on end and filled with water to a volume of 3.1 l (total capacity 4.4 l). Then oxygen was bubbled through this for 10 min to allow as much as possible to enter solution under normal atmospheric pressure. A randomly drawn sample of 5 goldfish was then placed in the water and the perspex end plate bolted on. With the chamber now set on its side, the air above the water was flushed out, with a powerful stream of oxygen, for one minute. With the outlet valve closed the pressure of the oxygen atmosphere above the water was then raised to whatever level was required.

Henry's Law states that the amount of gas which dissolves in a liquid is directly proportional to the partial pressure of the gas in the atmosphere above the liquid. The law was assumed to hold under the above experimental conditions, so that the tension of dissolved oxygen was computed from the recorded atmospheric pressure in the chamber (Fig. 2).

Apart from the higher oxygen concentrations obtained with this compression chamber it was also used to examine the influence on lethal temperature of various other concentrations of oxygen from a few per cent up to 400–500% of air saturation (Fig. 2). As in the first two series of experiments (Tables I and II) oxygen tensions of about 2 and about 4–5 atmospheres were obtained, without compression, merely by passing pure oxygen through the water. Tensions of oxygen less than air saturation were easily got by bubbling pure nitrogen through the water.

The aim in covering this very wide range of oxygen tensions (from near zero to about 30 atmospheres) was to test the influence of oxygen tension on thermal tolerance comprehensively. All oxygen tensions from 10–200% of air saturation were determined by means of a temperature-compensated oxygen electrode.

Lethal temperatures were determined by placing the chamber containing each batch of fish, immediately after the end plate was bolted on (and after compression if needed), into a previously prepared glass tank. For experimental series A (Fig. 2) the water in this tank was held at 41.5° C, and for series B (Fig. 2) at 43.1° C. These temperatures had been carefully predetermined as a result of pilot tests to discover the temperatures at which fish, acclimated at 17 and 27° C, would die. These tank temperatures were known to be sufficient to heat the water of the compression chamber past the lethal level, but to do it slowly enough for the accurate estimation of the temperature at death (movement cessation) for each fish. The fish could be easily seen through the perspex wall of the compression chamber. The chamber was not constantly lighted, but a lamp was positioned so that it could be used to examine the rear end of the chamber if fish drifted away from the perspex.

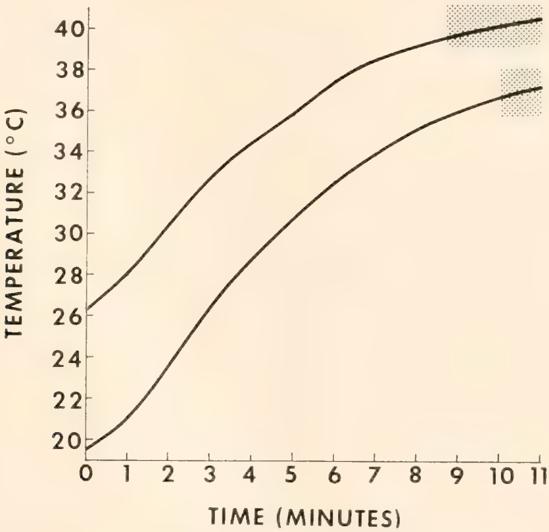

FIGURE 3. Rate of increase in temperature in compressor (Fig. 1) with external water temperature of 43.1° C (upper curve) and 41.5° C (lower curve). Hatching indicates range of temperature over which fish died in the experiment represented by each curve.

Figure 3 gives two curves depicting temperature rise against time in the chamber. The curves start at different values, each adjusted to the acclimation temperature of the animals. The curves pass through the actual temperatures recorded at one minute intervals: there has been no artificial smoothing.

It is noteworthy that rate of heating is not particularly significant in itself in influencing the temperature at which fish die. In several tests not reported here, the rate of heating to the lethal temperature was considerably more rapid than usual, though other conditions (i.e. oxygen tensions) were comparable. In these tests, mean lethal temperatures showed little difference as a result of the more rapid heating.

RESULTS

Oxygen-saturated water at normal atmospheric pressure

In experiments 1–5 plus several not reported here, the mean survival times of goldfish exposed to a lethal temperature in oxygen-saturated water, as compared to those in aerated water, were about 2–5 times greater (Table I). The difference between these means was significant at the 0.1% level ('t' test; Fisher, 1954).

While they remained alive, the gills of the fish exposed in oxygen-rich water were also visibly redder than those in ordinary water. Suggestive evidence only, this observation nevertheless accords with the idea that respiratory/circulatory failure accompanies progressive physiological impairment in the thermally stressed fish, *i.e.* that blood flow through the gills becomes reduced more rapidly in response to a relative oxygen shortage.

The results of experiments 6–10 (Table II), dealing with fish held at high oxygen tensions for various periods before exposure to the lethal temperature again showed a strong tendency for survival to be much longer than in ordinary aerated water. However, though the 'control' goldfish all died within a few minutes, the fish in oxygen-rich water showed a pronounced tendency to survive so much longer in all 'experimental' instances that each test was terminated with some fish still alive after 2 h. In testing the significance of the difference in survival times between 'experimental' and 'control' groups the Mann-Whitney non-parametric 'U' test was used instead of the 't' test (Siegel, 1956).

Results show that survivals in oxygen-rich water were all significantly better than in air-saturated water, but that fish placed directly in a high oxygen tension at the beginning of their 'lethal' test survived at least as well as those given longer periods in high oxygen tensions before testing. This disposes of the possibility that giving fish prolonged exposures to high oxygen *before* exposure to lethal temperature tests might cause them to survive even longer than those placed directly in high oxygen only at the beginning of the test.

In experiments 1–5 of Table I the pH in the control beakers was 7.5, and of the oxygen-rich ones 7.9; pH measured immediately before and after lethal tests showed no changes. Weights ranged from 0.4–1.3 g (mean 0.7 g) for 'calicoes' in experiments 1 and 2; from 0.3–1.1 (mean 0.7 g) for 'comets' in experiments 3 and 4; for experiment 5 the mean weight was 0.2 g.

In experiments 6–10 (Table II) pH of controls was 7.4 and in the oxygenated water was 7.6, whether after 2, $6\frac{1}{4}$ or $27\frac{1}{2}$ hr passage of oxygen. Weights of fish in this series ranged from 2.2–3.9 g (mean 3.0 g).

Higher concentrations of oxygen

Upper lethal temperature was obviously related closely to oxygen tension over a considerable range of values (Fig. 2). Mean lethal temperature increased in both series of experiments (*i.e.* with fish acclimated to 17 and 27° C, respectively) by about 2° C, from a low corresponding to oxygen tensions of about 10% of air saturation up to a maximum at approximately 5 atmospheres. This tension of oxygen is also about the same as that which produced highly significant increases in survival time in the first two series of experiments. Thus, increased ability to survive a high temperature because of oxygen abundance can be manifested either by increased survival time at a lethal temperature or by a higher mean lethal temperature achieved. There is nothing remarkable about these parallel associations; Fry (1967) has pointed out that they are only to be expected. It is, however, reassuring to find them demonstrated within one experimental study.

There was no additional enhancement of thermal tolerance in response to exposure to oxygen tensions in excess of 5 atmospheres—even up to approximately 30 atmospheres. The limit was reached quite abruptly. On the other hand, there was no evidence of oxygen toxicity effects at higher tensions.

In these experiments pH varied only over the range 7.4–7.5 in the compression chamber at the beginning of the tests. Weights in series A ranged from 2.9–4.4 g and in series B from 3.0–4.8 g.

Discussion

The results do not appear to be out of harmony with the hypothesis on which the experiments were based, so perhaps it may be seriously contended that some important malfunction of mechanisms that supply oxygen to the tissues begins at or near the lethal temperature level. Two arguments may be advanced in support of this. The first is that temperature tolerance is lowered when oxygen tensions are appreciably reduced below normal air saturation values; the second is that goldfish can survive for long periods in concentrations as low as 8% of air saturation at a temperature of 20° C (own unpublished observations). Blažka (1958) has shown that the closely related crucian carp *C. carassius* can live under conditions of almost if not complete anoxia for several months at low temperatures. So it seems that oxygen insufficiency is a relative problem for fish, exacerbated particularly when temperatures are dangerously high. This reasoning accords with the hypothesis of damage to respiratory, circulatory, and oxygen transport, systems. The fact that there is an increase in thermal tolerance in response to increased oxygen, of an order similar to the decrease in tolerance seen in oxygen-poor conditions, also supports the hypothesis—or certainly does it no violence.

On the other hand the suddenly achieved limit of this enhancement of tolerance suggests the operation of a new effect at an apparently very definite 'breakpoint', and here we may be witnessing a physiological failure of a quite critical sort. The enhancement of thermal tolerance up to 5 atmospheres of oxygen ends so abruptly that one must suppose not that more oxygen cannot be accepted by the tissues and body fluids of the fish, but simply that it is unable to utilize this extra oxygen. There might be several reasons for this and in concluding I list these.

(i) Heat denaturation of proteins including respiratory enzyme systems may make it impossible for the fish, as a whole organism, to make full use of the oxygen available to it.

(ii) Heat destruction of the lipoprotein of the cell membrane may make oxygen import into the cell, and/or carbon dioxide export out of it, impossible. At one time lipid was certaintly envisaged as important in thermal tolerance of goldfish (Hoar and Cottle, 1952a, b; Hoar and Dorchester, 1949).

(iii) Direct thermal death or irreversible damage of cells of the central nervous system may be quite unpreventable above a certain temperature maximum despite the presence of a superabundance of oxygen.

Any or all of the above effects may be at work; but perhaps some *system* critically linked to temperature may be responsible. If the latter proved correct it would be very interesting in several ways. One of these is a major point of this paper, which is to suggest that another system (or systems)—apparently failing at a critical level of temperature or after a critical exposure time—can have its failure alleviated merely by experimental amelioration of some accompanying environmental conditions. In that case it might be possible to postulate even more significant alleviations (if we could determine other ameliorating conditions for the new limiting factors).

SUMMARY

A hypothesis may be made out that the respiratory/circulatory, and possibly the oxygen transport, systems are importantly involved in thermal death of goldfish. Experiments showed that at two distinct levels of temperature acclimation, superabundant oxygen in the water could produce a definite improvement either in time of survival at a fixed lethal temperature or in lethal temperature reached as a result of heating.

Despite this positive effect of high oxygen a clear cut failure of oxygen, in excess of about 5 atmospheres (partial pressure), to produce further improvement in thermal tolerance suggests the failure of some critical system directly affected by temperature.

The experiments, while revealing nothing of the detailed mechanisms involved, certainly do not invalidate the hypothesis proposed, and open a way to further investigation.

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ACETYLCHOLINESTERASE ACTIVITY IN ESERINE-TREATED ASCIDIAN EMBRYOS

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Durante (1956, 1957) has shown histochemically that a cholinesterase is localized in the two lateral bands of muscle cells extending posteriorly in the larval tail of the ascidian, *Ciona intestinalis.* Since there was inhibition of normal muscle function in *Ciona* embryos and larvae exposed to eserine sulfate and other inhibitors of cholinesterase activity, the enzyme undoubtedly has a function in tail muscle contraction (Durante, 1958).

When *Ciona* embryos were treated with eserine during early embryonic stages, the inhibition of muscle activity which it caused was not reversible when the embryos were removed from the eserine solution, whereas, the inhibitory effect of eserine on fully developed tadpoles was reversible (Durante, 1958). Durante concluded that eserine may stop the synthesis of cholinesterase during development. The most interesting of the experiments she reports is a prefertilization effect of eserine. *Ciona* eggs placed in eserine for several hours prior to fertilization and subsequently fertilized and permitted to develop in eserine-free sea water, produced morphologically normal larvae which were quiescent, and which lacked sustained movements of the tail (Durante, 1958). This work supports the intriguing possibility that a specific enzyme inhibitor might prevent synthesis of the enzyme it inhibits.

The most plausible alternative to the explanation suggested by Durante is that eserine remains trapped in the egg or embryo, and that this residual eserine inhibits the muscular movement of the larva. However, it was the prefertilization experiment which led Reverberi (1961) to accept the conclusion that cholinesterase synthesis had been interfered with, because he considered it unlikely that the *Ciona* embryos could have retained eserine for such a relatively long time in pure sea water.

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In view of the potential importance of the Durante results, we have undertaken to repeat the prefertilization experiment and to examine the possibility of eserine retention. Our work unequivocally shows that eserine is trapped in the egg in sufficient quantity to inhibit most of the acetylcholinesterase activity in the developing embryo, and that acetylcholinesterase synthesis is not affected by the drug.

MATERIALS AND METHODS

Animals

Adult specimens of *Ciona intestinalis* L. were collected at Marina del Rey, California, and gametes were obtained surgically as described by Costello, Davidson, Eggers, Fox and Henley (1957). The embryos were reared in filtered sea water and at a constant temperature of 18° C ± 0.5° C.

Chemicals

Reagent grade chemicals were used throughout these studies. Eserine sulfate (H_2SO_4) USP was purchased from Calbiochem, BW62C47 [1:5-bis-(4-trimethyl-ammoniumphenyl) pentan-3-one diiodide] was a gift from the Burroughs-Wellcome Company, Tuckahoe, New York, and iso-OMPA (tetraisopropylpyrophosphoramide) was purchased from L. Light and Company Ltd., Colnbrook, England. Dithiobisnitrobenzoic acid was purchased from Aldrich Chemical Company; acetylthiocholine iodide and butyrylthiocholine iodide were obtained from Nutritional Biochemicals Corporation.

Preparation of homogenates for enzyme assay

Embryos of the desired stage were washed with filtered sea water, and collected by low speed centrifugation. One to five thousand embryos were homogenized in a variable speed motorized Potter-Elvehjem homogenizer with a teflon pestle in a medium consisting of 0.1 *M* sodium phosphate buffer (pH 8.0) with 0.1% Triton X-100 (Rohm and Haas, Philadelphia, Pennsylvania). Homogenates were centrifuged in an International Centrifuge Model V at 750 × *g* for 10 minutes, and the resulting supernatants assayed for acetylcholinesterase activity. Different homogenate supernatants contained 0.1–0.5 mg protein/ml.

Assay for acetylcholinesterase activity

A slightly modified method of Ellman, Courtney, Andres and Featherstone, (1961) was used to measure the acetylcholinesterase activity of homogenate supernatants (Fromson, 1968). Reaction mixtures contained 2.6 ml of 0.1 *M* sodium phosphate buffer (pH 8.0), 100 μl 0.1 *M* dithiobisnitrobenzoic acid in 0.1 *M* sodium phosphate buffer (pH 7.0), 100 μl 0.75 *M* acetylthiocholine iodide and 100 μl enzyme homogenate supernatant. The final substrate concentration in the reaction mixture was 2.5×10^{-2} *M*. This concentration was assumed to saturate the enzyme reaction based on the findings of Ellman *et al.* (1961). The reaction mixtures were incubated at 20° C in a water bath. Under these assay conditions, *Ciona* enzyme activity was directly proportional to incubation time and directly proportional to the amount of homogenate protein in the reaction mixture.

The enzyme reaction velocity studied was the change in optical density (Δ O.D.) at 412 nm in a Zeiss PMQII spectrophotometer. In Table I, where per cent activities of the enzyme were being compared, enzyme activity was expressed as Δ O.D. 412 nm × 10^3/min. Otherwise, In Table II and Figure 1, enzyme activity was expressed as a specific activity, millimicromoles substrate hydrolyzed/min/mg protein, since results with different homogenates were being compared directly. Four activity measurements were made on each homogenate used and the results expressed as a mean ± the standard error (S.E.) of the mean.

Protein analysis

Protein was precipitated from samples of enzyme homogenate by the addition of trichloroacetic acid to a concentration of 5%. An acid-insoluble fraction was prepared according to Whittaker (1966). The Lowry method was used to measure total protein in these acid-insoluble fractions (Lowry, Rosebrough, Farr and Randall, 1951) with crystallized bovine serum albumin (Armour Pharmaceutical Company) as a quantitative standard.

Eserine treatment

Unfertilized eggs were removed surgically from the oviducts of adult specimens of *Ciona* and washed in several large volumes of filtered sea water. Eggs from each adult organism were kept separately at 18° C for 90–120 minutes (more time than required for the first cleavage to occur). The eggs were examined microscopically and only batches of non-dividing eggs were used. These unfertilized eggs were pooled and divided into two groups. One group was held as a control. The other group was treated with 0.002% (3×10^{-5} *M*) eserine sulfate for one hour, and washed free of drug with an excess of filtered sea water. Control and eserine-treated eggs were then fertilized by addition of a dilute sperm suspension, and the resulting embryos reared in eserine-free sea water to the tadpole larva stage (18–19 hours at 18° C). There was no evidence that fertilization occurred in the control and eserine-treated eggs prior to the addition of sperm suspension.

RESULTS

Nature of the cholinesterase enzyme

Since the measured cholinesterase activity could be caused by a pseudocholinesterase as well as a true acetylcholinesterase, two series of experiments were performed which would distinguish between these two enzymes. Activities were measured after treatment of the enzyme preparation with three specific enzyme inhibitors, and in the presence of the cholinesterase substrates acetyl- and butyrylthiocholine. The results of these experiments are presented in Table I.

The cholinesterase inhibitor eserine sulfate (Chadwick and Hill, 1947) was added to larva homogenates (1.5×10^{-3} *M* final concentration). After a 20–30 minute incubation at room temperature, no enzyme activity was detected in this mixture. This rules out the possibility that a non-specific esterase is contributing to the cholinesterase reaction.

Larval cholinesterase was further characterized by the effect of specific enzyme inhibitors. Enzyme activity was measured in control homogenates, homogenates treated for 20 minutes with the pseudocholinesterase inhibitor iso-OMPA (tetraisopropylpyrophosphoramide, 10^{-3} *M*; Aldridge, 1953), and homogenates treated 20 minutes with the acetylcholinesterase inhibitor BW 62C47 [1:5-bis-(4-trimethylammoniumphenyl)pentan-3-one diiodide; Burgen, 1949]. The cholinesterase activity was inhibited 95.8% by BW 62C47 and only 5.5% by iso-OMPA. These data are consistent with the proposition that most of the enzyme activity is acetylcholinesterase activity.

TABLE I

Effect of substrates and specific enzyme inhibitors on the cholinesterase activity of embryo homogenates

Addition to reaction mixture	Function of additive	Enzyme activity (ΔO.D412 nm $\times 10^3$/min) mean ± S.E.	Per cent activity
Homogenate 1			
Acetylthiocholine iodide (2.5×10^{-2} *M*)	Cholinesterase substrate	34.10±0.36	100
Butyrylthiocholine iodide	Pseudocholinesterase substrate	1.53±0.12	4.5
Homogenate 2*			
None (control)		16.58±0.15	100
Iso-OMPA (10^{-3} *M*)	Pseudocholinesterase inhibitor	15.68±0.12	94.5
BW62C47 (10^{-3} *M*)	Acetylcholinesterase inhibitor	0.70±0.01	4.2
Eserine sulfate (1.5×10^{-3} *M*)	Cholinesterase inhibitor	0	0

* Acetylthiocholine iodide (2×10^{-2} *M*) used as substrate.

Acetylcholinesterase and pseudocholinesterase are also distinguished by their respective substrate specificities. Since acetylcholinesterase is highly specific for acetate containing substrates (Aldridge, 1953), enzyme activities were measured using the substrates acetyl- and butyrylthiocholine iodide. The measured enzyme activity with the butyrylthiocholine was only 4.5% of the activity measured using the acetylthiocholine iodide substrate. Therefore, the enzyme activity measured in tadpole homogenates is attributable to the presence of acetylcholinesterase.

Effects of eserine treatment

Unfertilized eggs were treated with 3×10^{-5} *M* eserine sulfate and washed free of the drug after one hour. These eggs were then fertilized and reared in eserine-free sea water. The resulting larvae exhibited a vastly reduced capacity for movement. Despite this striking physiological impairment, no morphological irregularities were observed upon microscopic examination of these larvae. These findings are similar to those reported by Durante (1958).

Acetylcholinesterase activity, measured in homogenates of larvae hatched from eserine-treated eggs (as described above), was inhibited 80–87% (Table II and

Fig. 1). These quantitative measurements of enzyme activity confirm Durante's (1958) hypothesis that embryos treated with eserine prior to fertilization are defective in acetylcholinesterase activity.

Eserine retention

Eserine accumulation and retention during the one hour prefertilization incubation is indicated by the following results. Acetylcholinesterase activity assayed in homogenates of larvae hatched from eserine-treated eggs was inhibited 80% (Table II). Homogenate mixtures containing equal amounts of control and eserine-treated embryo homogenates should have a predictable enzyme activity calculated by averaging the mean activity values for each of the two component homogenates.

Table II

Acetylcholinesterase activity of embryos treated prior to fertilization with eserine

Embryo homogenates	Acetylcholinesterase activity (mμmoles substrate hydrolyzed min mg acid-insoluble protein) mean ± S.E.
Control	32.63 ± 0.57
Eserine-treated	6.60 ± 0.32
Calculated activity of a 1:1 mixture of control and eserine-treated embryo homogenates	19.61
Actual activity in a 1:1 mixture of control and eserine-treated embryo homogenates	6.23 ± 0.09

In the experiment reported in Table II, this calculated level was 56% of the control enzyme activity level. However, the actual acetylcholinesterase activity detected in the homogenate mixture was only 17% of the control level. Apparently, unfertilized *Ciona* eggs treated with eserine accumulate and retain this drug in sufficient quantities to inhibit the majority of the acetylcholinesterase activity in the homogenate mixture. In fact, the acetylcholinesterase activity in the mixed homogenate was not significantly different from the activity measured in the eserine-treated embryo homogenate alone.

Restoration of enzyme activity

If the sole action of the accumulated eserine is to inhibit enzyme activity, then the presence of this inhibitor during development should not interfere with acetylcholinesterase synthesis. Removal of the inhibitor from homogenates of prefertilization-treated embryos should restore acetylcholinesterase activity to the level of control homogenates. Eserine is a reversible inhibitor of acetylcholinesterase activity; it can easily be removed from the enzyme by dialysis (Cohen, Kalsbeek, and Warringa, 1948).

Homogenates were prepared from control and experimental larval stage embryos and acetylcholinesterase activity measured. The experimental embryos were treated with 3×10^{-5} M eserine sulfate for one hour prior to fertilization. In the experiment shown in Figure 1, eserine treatment resulted in an 87%

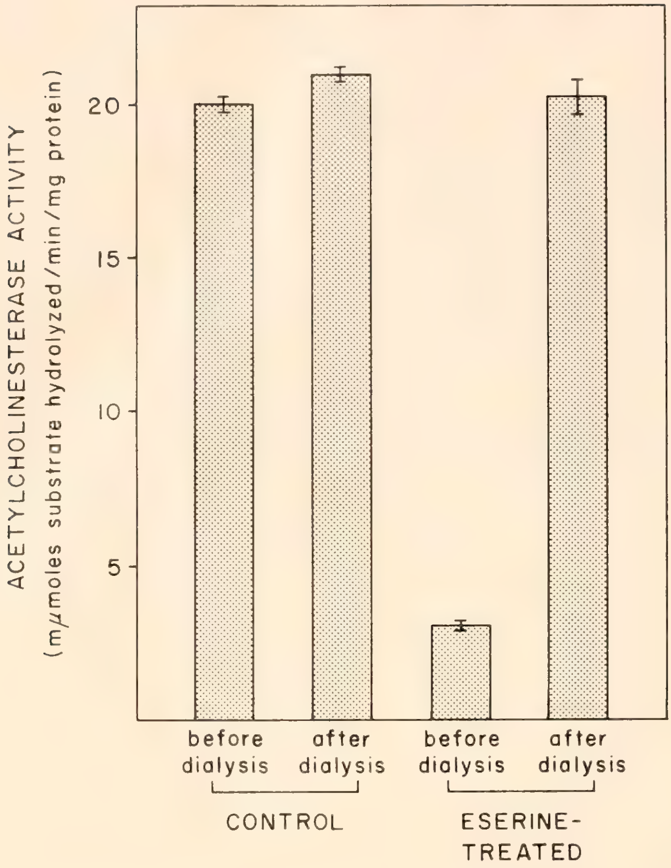

FIGURE 1. Unfertilized *Ciona* eggs incubated in 3×10^{-5} *M* eserine sulfate for 1 hour prior to fertilization. The eggs were washed free of eserine, fertilized, and allowed to develop into larvae. Acetylcholinesterase was measured in control and eserine-treated larva homogenates. These two homogenates were then dialyzed against distilled water, and the post-dialysis enzyme activities of control and eserine-treated embryo homogenates measured. The bars represent the mean enzyme activity of 4 activity measurements on each homogenate; the vertical lines indicate the standard error of the mean.

inhibition of enzyme activity. These control and eserine-treated embryo homogenates were dialyzed against distilled water for 55 hours at room temperature. After dialysis, the enzyme activities in the homogenates were determined once again. Eserine inhibition was completely reversed by dialysis; acetylcholinesterase activities in the dialyzed control and the dialyzed experimental embryo homogenates were equal (Fig. 1). Since dialysis restores the level of enzyme activity in the experimental homogenate to that found in the control homogenate, the presence of eserine has obviously not prevented the synthesis of a full complement of acetylcholinesterase in these embryos.

An interesting point concerning the stability of *Ciona* acetylcholinesterase is illustrated by the dialysis of control homogenate in Figure 1. Since there is no loss of activity following a dialysis of two days duration at room temperature, the enzyme is shown to be remarkably resistant to denaturation. This is also true for acetylcholinesterase from other animal sources (Nachmansohn and Wilson, 1955).

DISCUSSION

Sawyer (1943) has shown clearly that eserine does not prevent synthesis of cholinesterase in developing *Amblystoma* embryos. Therefore, there is little reason to accept the conclusion that Durante (1958) reaches from her experiments with *Ciona* embryos, namely, that eserine has interfered with the synthesis of cholinesterase. Unlike Sawyer, Durante has no supporting evidence from enzyme studies; her conclusion is based solely on the irreversible inhibition of larval motility caused by eserine treatments. The prefertilization experiment with eserine provides the best circumstantial evidence that enzyme synthesis may be deficient, because, as Reverberi (1961) points out, eserine would have to be retained in the egg for an unusually long time if trapped eserine is the real cause of the inhibited motility.

We know of no evidence from any biological system that a specific enzyme inhibitor which is not also a substrate analog could possibly prevent synthesis of an enzyme. On further investigation we have discovered that the Durante work is no exception. There is enough eserine trapped in *Ciona* embryos following prefertilization treatment to inhibit the acetylcholinesterase of control embryos when homogenates of both are mixed together. Full acetylcholinesterase activity can be restored in homogenates of experimental embryos by simple dialysis. All of the Durante results can be explained by retention of eserine in the egg or embryo following even a brief exposure to the drug.

Based on the Durante (1958) experiments, eserine seems to be freely diffusible into the egg at any stage of development and retained by all the pre-hatching stages. Since reversal of the reduced mobility caused by eserine occurred only in larvae which were first treated with the drug after hatching, it seems likely that the egg membranes are a permeability barrier which restricts loss of eserine in eserine-free sea water. This question could be studied further using enzyme preparations which de-chorionate ascidian eggs (Berrill, 1937).

Our investigation also confirms an important observation made by both Sawyer (1943) and Durante (1958). Differentiation of the morphological structure as well as the potential physiological function of an acetylcholinesterase-containing tissue is independent of the functional activity of the enzyme during development. Likewise, the function of tyrosinase in ascidian pigment cells is not necessary for normal differentiation of the cells (Minganti, 1957; Whittaker, 1960, 1966). This principle probably applies to the specialized enzyme systems of most differentiating cells and tissues.

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SUMMARY

1. The use of specific cholinesterase inhibitors and substrates demonstrated that the enzyme activity in *Ciona intestinalis* larvae is an acetylcholinesterase.

2. Eggs treated with eserine sulfate (an acetylcholinesterase inhibitor) for one hour prior to fertilization developed into larvae with defective muscular movements and greatly reduced levels of acetylcholinesterase activity.

3. Two kinds of experiments show that this reduced enzyme activity was caused by the retention of eserine and not by inhibition of acetylcholinesterase synthesis. Homogenates of embryos from eserine-treated eggs inhibit acetylcholinesterase activity when mixed with homogenates of control embryos. Full enzyme activity in homogenates of the experimental embryos could be recovered by dialysis.

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FILTRATION OF PARTICLES FROM SUSPENSION BY THE AMERICAN OYSTER *CRASSOSTREA VIRGINICA*[1]

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The oyster *Crassostrea virginica* is a suspension feeder which retains suspended particulate material on its gills. These are lamellar and composed of rows of filaments in groups that constitute folds (plicae) in the lamellar surface (Nelson, 1960). Water is pumped through small interfilamentary openings (ostia) by the action of cilia on the filaments. The actual manner in which particles are retained on the gills is not completely understood (Jørgensen, 1966). Suggested mechanisms include entrapment in mucus, straining action of large latero-frontal cilia, and limitation of passage through the gills by the size of the interfilamentary ostia.

Two methods have been commonly used to study particle filtration by bivalves. One involves channeling water into and from shell cavities with devices which collect all water flowing through the gills (Galtsoff, 1928; Loosanoff and Engle, 1947; Tammes and Dral, 1955). These studies have been criticized because oysters may not behave normally when subjected to the stress of the collecting device. The second involves measurements of rates at which undisturbed lamellibranchs clear particles in standing water. This latter method has been used extensively by many workers in recent years (Jørgensen, 1949, 1960; Ballantine and Morton, 1956; Chipman and Hopkins, 1954; Jørgensen and Goldberg, 1953; Willemsen, 1952). Objections to studies in standing water are that previously filtered material may be resuspended and refiltered (recycled) and also that particle concentrations will change with time. With only several exceptions, as exemplified by the work of Loosanoff and Engle (1947), particle density was measured indirectly by techniques such as isotope labeling, per cent light transmission, *etc.* Results of these studies differ widely in respect to the efficiency at which various size particles are filtered from suspension. No one has correlated the results of these investigations with the structures and mechanisms which determine them (Jørgensen, 1966).

The purpose of this investigation was to establish more precisely the size of the smaller particles removed from suspension by the American oyster *Crassostrea virginica* and the efficiency of removal and to relate our results to the most probable mechanism involved.

Materials and Methods

Apparatus and materials

The basic design of the study was to hold oysters in troughs of flowing water and to measure differences in numbers of particles entering and leaving the system. Differences were attributed to filtration by the animals. The experimental ap-

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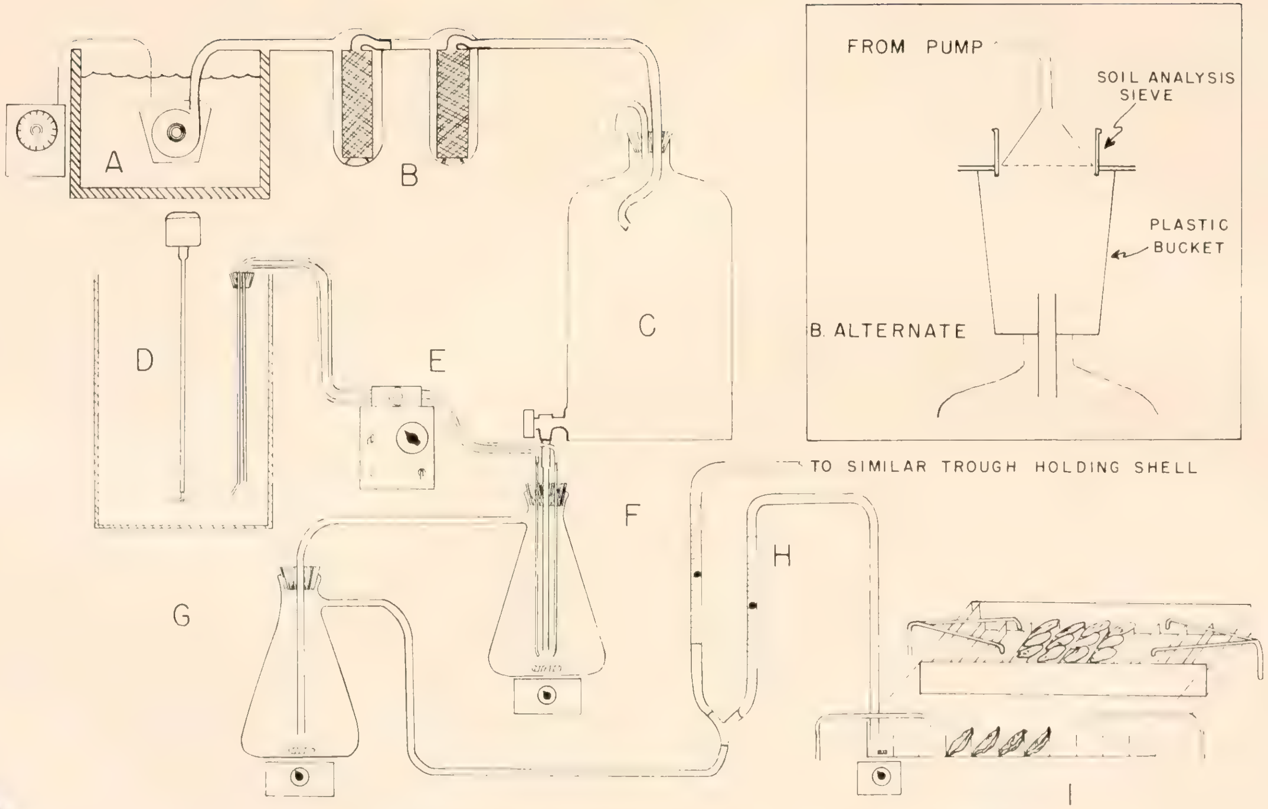
A
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FROM PUMP
SOIL ANALYSIS SIEVE
PLASTIC BUCKET
B. ALTERNATE
TO SIMILAR TROUGH HOLDING SHELL

FIGURE 1. Diagram of open flow system used in particle filtration experiments. Explanation of apparatus appears in text.

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proach of our study differed from previous studies in several respects. Particle filtration was studied simultaneously over a continuous spectrum of sizes ranging from about 1.0 μ to 12.0 μ, and direct measurements and counts of the particles involved were made. Water flowed continuously over the oysters under conditions approaching those of the natural environment. This also served to avoid recycling. Filtration activity was not measured instantaneously but covered a period of approximately 12 min.

The experiments were conducted at the Virginia Institute of Marine Science, Gloucester Point, Virginia. Preliminary studies began in 1964 and 1965, but most data were obtained during 1966 and 1967.

Water for all studies was pumped through plastic pipes from a source 0.5 m above the bottom, 90 m offshore in the York River, to a constantly overflowing overhead trough in the laboratory. Water flow in 1966 experiments was 1.01 l/min; in 1967, flows were regulated to range from 0.19 to 1.01 l/min.

Data were collected using a system having continuous water flow over the oysters and controls (single oyster shells). Particle removal was studied in respect to 1) the natural particle complement of York River water (in one series of experiments) and 2) kaolinite clay particles added to filtered York River water. Kaolinite (American Petroleum Institute standard no. 7, Bath, South Carolina) with a density of 2.6 was similar to the mineral components of suspended matter in the York River (Nelson, 1963).

The apparatus used is shown in Figure 1. Water was pumped intermittently from an overhead trough (A) through two spool-wound yarn depth filters (porosity of 20 μ and 1 μ, respectively) in series (B) into a 13-gal polyethylene carboy (C). An alternate filtration system (B Alternate) was used in series with river water without added materials. In these experiments a standard soil analysis sieve with mesh size of 44 μ was substituted for the depth filters. Passage through the soil analysis sieve did not appreciably change quantity of suspended solids since it had been found previously that 97% by weight of the suspended material in the York River passes through a 44 μ sieve (Haven and Morales-Alamo, 1968). The carboy served to maintain a relatively constant water head and to enhance dissipation of air bubbles.

Kaolinite particles added to the flowing water were maintained in suspension in an acrylic tank (D) with an electric stirrer. From this source they were metered with a peristaltic pump (Harvard Apparatus Co., Dover, Massachusetts) into a 4,000-cc mixing flask (F). A magnetic stirrer mixed the suspensions with the filtered water and a second 4,000-ml flask (G) in series with the first enhanced homogeneity.

The mixture passed through flow meters (H) into two identical acrylic plastic experimental troughs (I) holding live oysters and oyster shells, respectively; troughs were 47 cm long by 13 cm wide and 6 cm high. A small magnetic mixing bar at the head of each trough under the inflowing hose further mixed the inflowing suspension. Three oysters (or shells) were placed side by side in each of four successive compartments. Twelve oysters were used in all studies except several in 1967 which used 24. Water depth above oysters and shells was approximately 2 cm.

Oysters came from an area free of known oyster diseases (Horsehead Rock) in the James River, Virginia. They were about 2 years old and averaged 50 mm in height with a mean wet meat weight of 4.8 g. To minimize possibility of particle recycling, flows and oyster numbers were chosen so that flow exceeded the theoretical pumping capacity of the oysters. Calculations showed that each oyster received a flow of 1.05 l/hr per gram of wet meat weight. Jørgensen (1966) summarized data showing that adult oysters may pump through their gills from 0.5 to 0.8 l/hr per gram of wet meat weight.

Oysters and shells were scrubbed, rinsed, and immediately placed in experimental troughs enclosed in a large, darkened box.

Total solids were measured by filtration through 0.45 μ membrane filters. Salinities and temperatures were monitored daily during the experimental periods with a stem hydrometer and a Foxboro recording thermometer, respectively. During 1966, studies extended from 24 June to 14 October, during which period salinities ranged from 18.0 to 24.1 ‰ and temperatures from 17.9 to 28.0° C. In 1967, experiments were conducted from 10 May to 12 June, with temperatures ranging from 14.2 to 23.4° C and salinities from 19.0 to 20.2 ‰.

Collection of samples

Samples of water for particle size analysis were collected simultaneously from two pairs of 3-mm I.D. glass tubes which siphoned water continuously from the troughs halfway between the water surface and the top of the compartmental baffles (Fig. 1). One pair of tubes was located over the compartment immediately preceding the oysters (or shells), a second pair over an empty compartment at the outlet. Samples were not collected until oysters were actively pumping and producing faeces or pseudofaeces, a period ranging from $\frac{1}{2}$ to 2 hr. Faeces or pseudofaeces produced by the oysters settled to the bottom of the troughs without contributing to the particle load flowing from the system. Each sample to be analyzed for particle size was obtained by combining five subsamples, each of which took about 30 sec to collect and was obtained at 3-min intervals over a 12-min period. Consequently, each sample represented oyster activity over a 12-min interval.

In many experiments it was necessary to dilute samples to retard flocculation and reduce inaccurate counts resulting from coincident passage of particles through the tube orifice of the Coulter Counter. York River water, filtered twice through 0.45 μ membrane filters, was used as dilutant. Subsamples collected from the troughs were diluted immediately by pouring into a flask containing a predetermined volume of dilutant. Dilution factors ranged between 1.9 and 3.6, depending on particle concentrations flowing through the troughs. After collection, samples were held at 26° C in a constant temperature water bath until counted since it was earlier found that changes in sample temperature affected the counts recorded by the Coulter Counter.

Coulter Counter and analysis of samples

Particles in the water samples were measured and counted using a Model B Coulter Electronic Particle Counter (Coulter Electronics, Inc., Franklin Park, Illinois). The counter principle is based on particles suspended in an electrolyte being forced through a small orifice between two electrodes through which an electric current path has been established; each particle displaces electrolyte in the orifice essentially equal to its volume and creates a change in current resistance proportional to this volume (Kubitschek, 1960; Sheldon and Parsons, 1967). The counter does not measure linear dimension, and particle diameter is calculated from the displaced volume and represents the diameter of a sphere having the same volume as the particle. Particle counts are recorded on a decade counter.

Cumulative particle counts made at increasing consecutive particle size settings were processed through an IBM 360 computer. Counts were corrected for coincident passage through the orifice, for background count in the dilutant water, and for dilution, and were then converted to differential counts. The latter were subsequently converted to total volume of all particles in the size interval. Size intervals were 1.0 μ in most instances; however, on several occasions intervals ranging from 0.1 to 0.5 μ were used for sizes near 1.0 μ. In several instances intervals of 2.0 μ were used for sizes larger than 5.0 μ. Efficiency of removal of particles is expressed as per cent difference in numbers of particles of a size interval between the inflowing and outflowing water samples.

TABLE I

Mean and range of number of particles of different size fractions per liter of water entering troughs with oysters in 1966 series of experiments

Size fraction (μ)	No. replications	No. of particles (millions)	
		Mean	Range
		Natural particles	
1.00–1.99	3	1001.4	790.4–1230.6
2.00–2.99	3	103.2	77.3–131.0
3.00–3.99	6	33.3	20.4–48.3
4.00–4.99	6	10.9	6.4–14.0
5.00–5.99	6	5.5	4.2–7.6
6.00–6.99	5	2.3	1.6–2.8
7.00–7.99	4	1.1	1.1–1.3
9.00–9.99	3	0.3	0.3–0.4
11.00–11.99	3	0.1	0.1–0.1
		Kaolinite particles	
1.00–1.99	34	921.2	391.7–1756.0
2.00–2.99	34	125.8	65.1–492.1
3.00–4.99	34	59.2	16.2–118.0
5.00–6.99	12	8.9	4.5–16.7

Data collected in preliminary studies conducted in 1964 and 1965 were subject to excessive variations in the control data and are presented only as a supplement to data obtained in 1966 and 1967. Later modifications eliminated this problem (see Methods).

The data on per cent removed were compared by analysis of variance after being transformed into degrees using the arcsin percentage transformation. Further analysis was made using Scheffé's multiple mean comparison method (Guenther, 1964). Data are presented graphically in terms of the point and 95% interval estimate of the mean, $\bar{x} \pm S_{\bar{x}} t_{0.05}$, where $\bar{x}$ = mean, $S_{\bar{x}}$ = sample standard error, and $t_{0.05}$ = the 5% level of student's t distribution (Snedecor, 1956).

RESULTS

Individual experiments with kaolinite and natural particles showed a consistent pattern. There was a sharp increase in per cent of particles removed as particle size increased from 1.0 to between 2.0 and 4.0 μ. At larger sizes the per cent removed leveled off with no indication of further increase in efficiency.

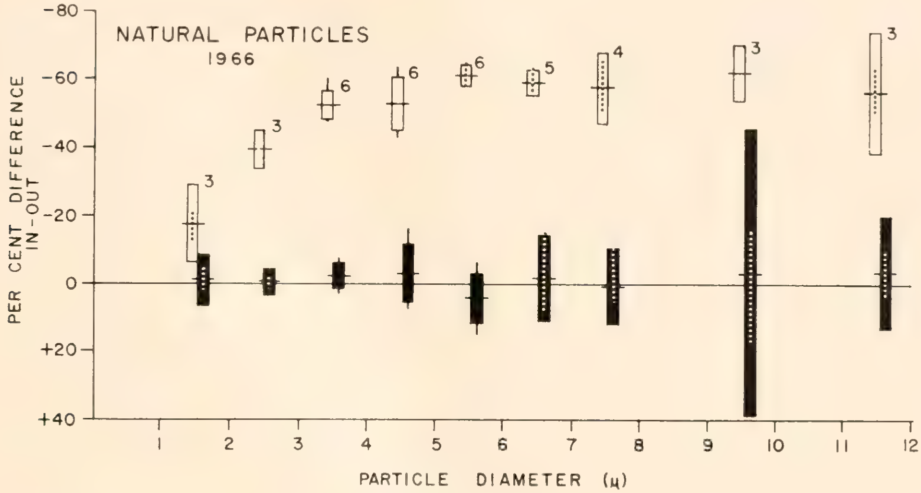

FIGURE 2. Mean per cent difference between particle numbers in water entering and water leaving experimental troughs for separate size fractions in 1966 natural particles experiments, plotted at mid-point of size interval; open bars = oysters, solid bars = shells; horizontal line = mean, vertical line = range, bar = interval estimate ($\bar{x} \pm S_{\bar{x}} t_{0.05}$). Figures over bars indicate number of replications.

Variations were present between individual experiments, especially in those
using kaolinite particles, but the general pattern described above appeared con-
differences in the filtration activity of the oysters at the time a particular experiment
was conducted. Insofar as temperature is concerned, Loosanoff (1958) observed no
significant change in pumping rate of oysters between 16 and 28° C. In the
absence of any observed relation for these three parameters, data for particle
sistently in every experiment. These variations could not be correlated to differences
in temperature, salinity or particle concentration and probably resulted from
removal were combined for similar size intervals. Results of studies made at
size intervals different from those included above are reported separately. During
most studies, oysters produced faecal strings and pseudofaeces, indicating filtration
of particles from suspension and ingestion of some.

Filtration under flow of 1.01 l/min using 12 oysters

Filtration of natural particles was studied in 1966 using 12 oysters and a flow of 1.01 l/min. A total of six studies was combined into a composite curve at intervals of 1.0 μ (Fig. 2).

Particles in the undiluted water entering the trough were by far the most abundant in the 1.0 to 2.0 μ size range where an average of over 1 billion per liter was measured. Numbers decreased rapidly with size and only 70,000 per liter were measured in the 12.0 to 13.0 μ size range (Table I). Total solids were not consistently measured; however, during July and August 1966 in the York River they ranged from 4.7 to 29.0 mg/l, with a mean of about 10.0 mg/l (Haven and Morales-Alamo, 1966a).

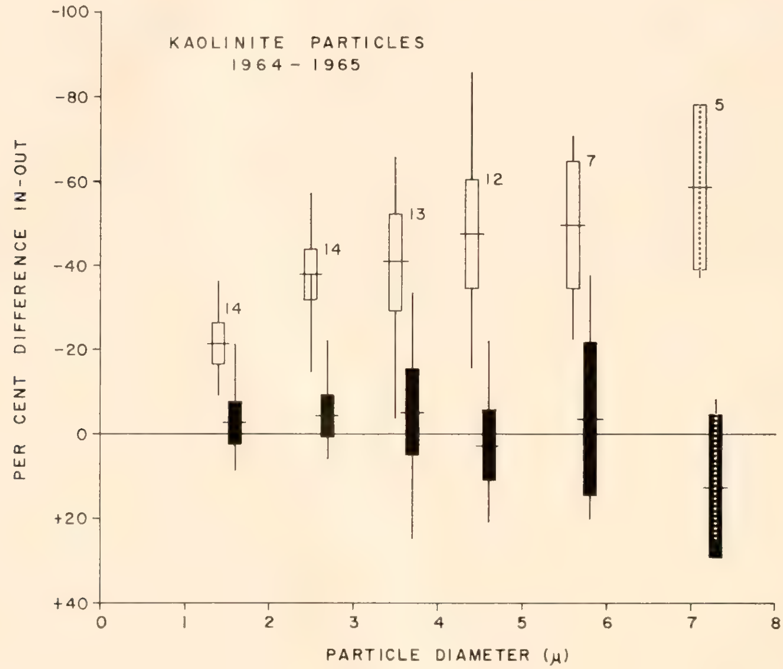

FIGURE 3. Mean per cent difference between particle numbers in water entering and water leaving experimental troughs for separate size fractions in 1964–1965 kaolinite particles experiments; open bars = oysters, solid bars = shells; horizontal line = mean, vertical line = range, bar = interval estimate ($\bar{x} \pm S_{\bar{x}}t_{0.05}$). Figures over bars indicate number of replications.

Oysters removed a mean of 18% of the particles between 1 and 2 μ. Average removal increased to 53% between 3.0 and 4.0 μ. Above this size, there was no evidence of further differences in efficiency of removal up to 12.0 μ. Mean per cent differences between inflow and outflow in the controls were close to zero with a small variance for most size intervals. There was no evidence of a difference among these means.

Two separate studies conducted in August 1966 using natural particles with 12 oysters and a flow of 1.01 l/min included size intervals under 1 μ. In the first, oysters removed 34.8% of the particles between 0.8 and 0.9 μ and 37.8% of the particles between 0.9 and 1.0 μ. The second experiment showed a removal of 28.5% for particles between 0.9 and 1.0 μ.

Data on filtration of kaolinite particles obtained during preliminary studies conducted in 1964 and 1965 showed a similar pattern of removal with increase in particle size (Fig. 3). Removal efficiency increased from 21% between 1.0 and 2.0 μ to 38% between 2.0 to 3.0 μ; there was no evidence of further change in efficiency at larger sizes. The values recorded in these data are not considered reliable because of the excessive variation observed in the data but are included here only to illustrate the recurrence of a plateau in removal efficiency at particle sizes larger than 3 μ.

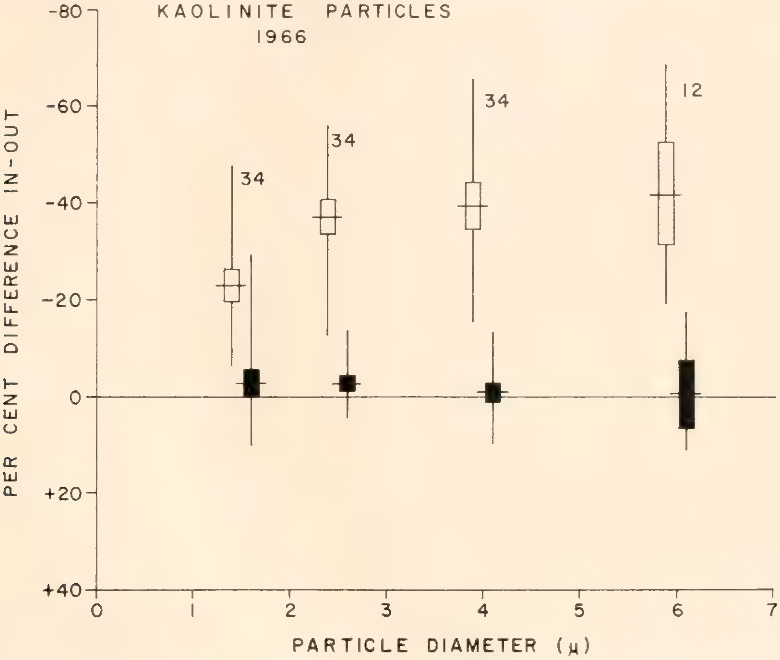

FIGURE 4. Mean per cent difference between particle numbers in water entering and water leaving experimental troughs for separate size fractions in 1966 kaolinite particles experiments; open bars = oysters, solid bars = shells; horizontal line = mean, vertical line = range, bar = interval estimate ($\bar{x} \pm S_{\bar{x}} t_{0.05}$). Figures over bars indicate number of replications.

Data on filtration of kaolinite particles in 1966 were obtained by combining 34 separate studies using a flow of 1.01 l/min and 12 oysters. Particles over 7.0 μ in the diluted samples were too few for precise counts and were not included in the analysis.

Particles in the undiluted inflowing water were most numerous in the 1.0 to 2.0 μ size range where they averaged 921 million per liter. Particle number decreased to 8.9 million between 5.0 and 6.0 μ (Table I).

Removal of particles between 1.0 and 2.0 μ averaged 24% (Fig. 4). This increased to 37% for 2.0–3.0 μ particles and reached 42% between 5.0 and 7.0 μ. There was no evidence of a significant difference in mean per cent removed among the three particle size fractions included between 2.0 and 7.0 μ. In the controls, mean differences in particle counts between inflow and outflow were close to zero, with only small variances for all size intervals. As in studies with natural particles, there was variation between rates of removal at various particle sizes. However, most of the studies fell within relatively narrow limits.

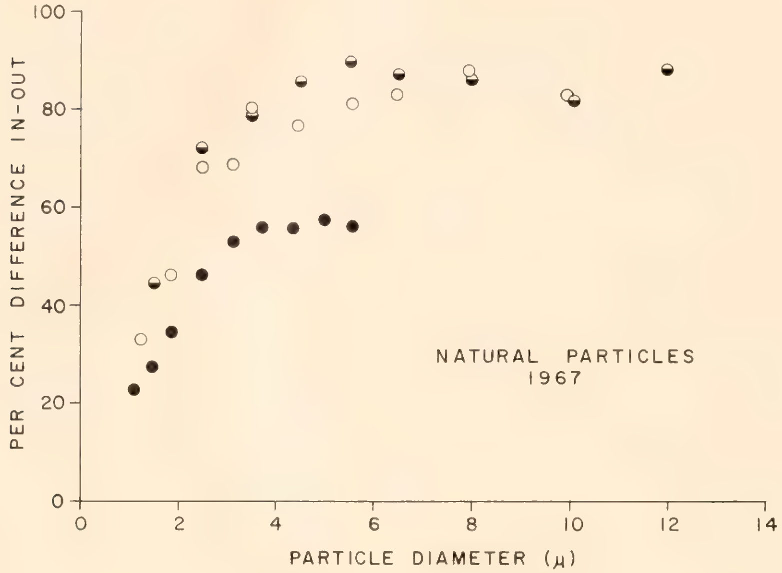

FIGURE 5. Mean per cent difference between particle number in water entering and water leaving experimental trough with oysters for separate size fractions in 1967 natural particles experiments, plotted at mid-point of size interval; solid circles = 12 oysters, flow of 1.01 l/min; open circles = 12 oysters, flow of 0.19–0.42 l/min; half-filled circles = 24 oysters, flow of 0.83–1.01 l/min.

Two of the experiments with kaolinite particles conducted in August 1966 included counts made at intervals smaller than 1.0 μ. These showed an average per cent removal of 32.7% for particles between 1.1 and 1.5 μ, 43.9% for particles between 1.5 and 2.0 μ, and 54.2% for particles between 2.0 and 2.5 μ.

Filtration under variable flow and oyster number

It was possible that the basic pattern of particle removal for all preceding data was a direct result of the particular experimental conditions used, *i.e.*, a flow of 1.01 l/min and 12 oysters. To test this possibility, experiments with natural particles were conducted in May 1967 at various flow rates and with 12 or 24 oysters.

Per cent removal using the standard 1.01 l/min flow with 12 oysters increased from 23% for particles between 1.0 and 2.0 μ to 56% between 3.0 and 4.0 μ. No change in per cent removed was evident for sizes above 4.0 μ (Fig. 5, solid circles). These data were almost identical to data collected in the 1966 experiments. Seven experiments were conducted at flows of 1.01 l/min but with 24 oysters. Doubling oyster number displaced the entire curve upward. The basic pattern, however, remained the same (Fig. 5, half-filled circles), with efficiency increasing in the 1.0 to 4.0 μ range and then leveling off at about 88%. In effect, doubling oyster number nearly doubled rates of removal. A reduction in flow but with 12 oysters had nearly the same effect as doubling oyster number and the basic pattern was still evident (Fig. 5, open circles).

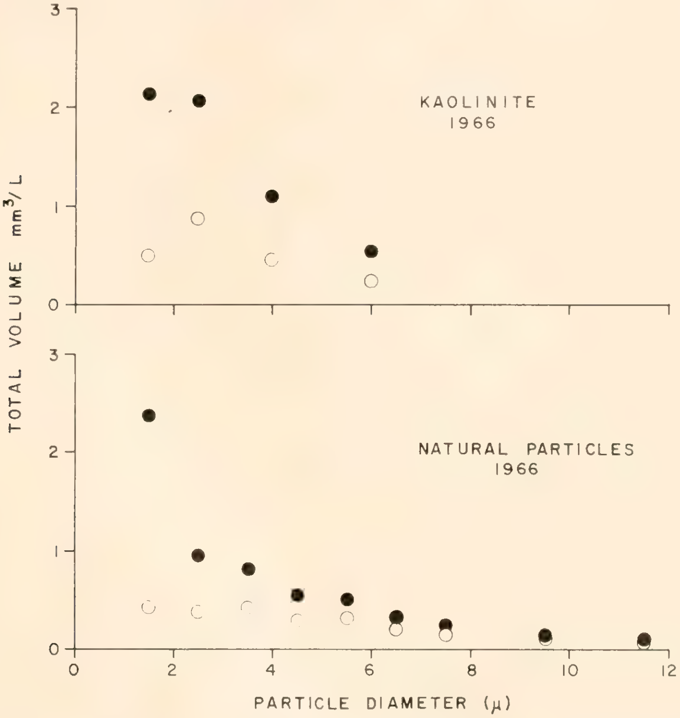

FIGURE 6. Mean total volume of particles in the different size fractions entering and leaving experimental troughs with oysters in 1966 kaolinite and natural particles experiments, plotted at mid-point of size interval; closed circle = entering, open circle = leaving.

Particle removal in terms of volume

It was shown previously that natural particles were numerically most abundant between 1.0 and 2.0 μ and decreased to much lower levels between 11.0 and 12.0 μ. A similar relationship was found when particle numbers were converted to volumes (Fig. 6). Mean total volume for particles between 1.0 and 2.0 μ entering the system was about 2.5 times greater than in either of the next two larger size fractions and decreased significantly with particle size in the size range included. A test on the slope of the regression of mean total volume removed on particle size showed that it was significantly different from 0 ($P < 0.05$). Therefore, volume removed decreased with increase in particle size ($r = 0.971$). The mean volume removed by oysters between 1.0 and 2.0 μ was 0.425 mm^3, but only 0.066 mm^3 between 11.0 and 12.0 μ. The combined volume removed between 1.0 and 4.0 μ represented 52.0% of the total removed between 1.0 and 12.0 μ; that removed between 7.0 and 12.0 μ represented much less, being 13.5% of the total. The 4.0 to 7.0 μ fraction represented 34.5% of the total.

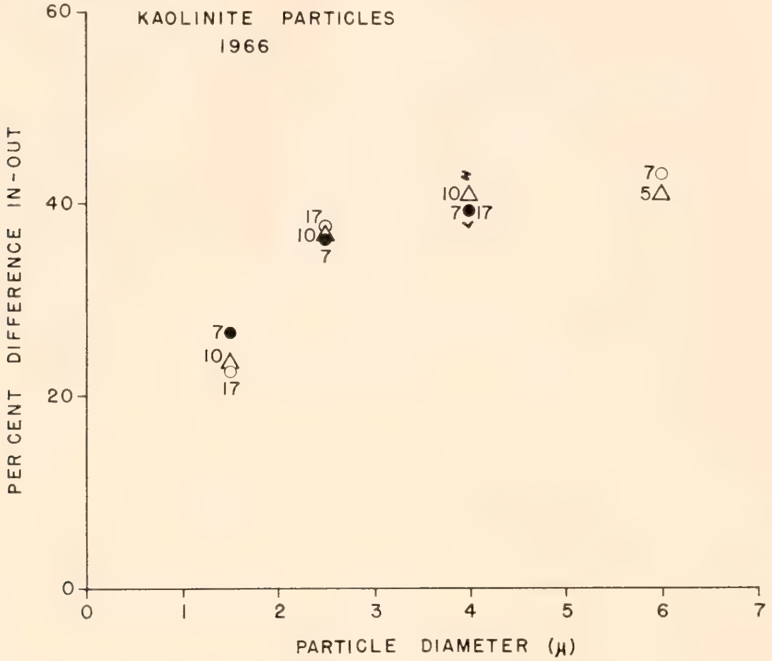

FIGURE 7. Mean per cent difference between particle numbers in water entering and water leaving experimental troughs for separate size fractions in 1966 kaolinite experiments. Data grouped into three intervals representing low, moderate and high particle concentrations in inflowing water; solid circles = 5.91–10.10 mg/l; open circles = 11.40–19.60 mg/l; open triangles = 20.34–37.66 mg/l. Figures represent number of replications.

Conversion of kaolinite particle counts to volume also showed a greater mean total volume of particles between 1.0 and 2.0 μ than of any of the other size fractions entering the system (Fig. 6). Volumes plotted at the mid-points for the 3.0–5.0 and 5.0–7.0 μ intervals are one-half of the total volumes in the 2.0 μ intervals. That is, the value plotted represents mean removal rates for a 1.0 μ interval.

The data for volumes of kaolinite particles removed were distributed differently than those for natural particles. Although a relatively high correlation coefficient of $r = 0.751$ was obtained, further analysis showed no evidence that the regression slope of mean total volume on particle size was significantly different from 0 ($P > 0.05$). Therefore, the data do not show that volume removed decreased with increase of particle size. They do show, however, that as much volume between 1.0 and 2.0 μ was removed as of any other size fraction.

The preceding results show that volumes of suspended material removed by oysters in the vicinity of 1.0–3.0 μ are at least as great as those in larger size intervals measured when considered in terms of the total volume rather than in terms of the efficiency with which oysters remove them.

Volume data from individual experiments with kaolinite particles, conducted in 1966, were used to illustrate the absence of a relation between concentration of particles entering the system, expressed as mg/l, and per cent of the particles removed. For this purpose, the data were converted into mg/l by multiplying total volume of all particles in the size spectrum included in each experiment by 2.6, the approximate density value for kaolinite. These data were then grouped into three intervals representing low, moderate and high concentrations. These were, respectively, 5.91–10.10, 11.40–19.60 and 20.34–37.66 mg/l (Fig. 7).

Results showed that, within the size and volume limits studied, the quantity of material present in the inflowing water did not affect the per cent of particles removed at any of the size intervals measured or the shape of the curve for per cent removed *vs.* particle size.

DISCUSSION

Jørgensen (1960) states that results obtained by different investigators often vary or even disagree and that quantitative data on feeding in lamellibranchs would be of greater value if they can be shown to have been derived from experiments on undisturbed animals. We consider that our experimental technique approached the undisturbed conditions of the natural environment.

Other workers have found a relation similar to that shown by our data between particle size and per cent removal for *C. virginica* while the data of others disagree. Jørgensen and Goldberg (1953) found that graphite particles of sizes down to a few microns were effectively retained by *C. virginica* and that 80% of the 1.0 to 2.0 μ particles passed through the gills. Loosanoff and Engle (1947) showed that cells of the flagellate *Euglena* 60 μ in length easily passed through the gills of *C. virginica* and sometimes only 15% was removed from suspension. In the 5.0 μ size they found oysters retained 0 to 85% of the *Chlorella* sp. cells. They concluded that little correlation existed between size of the microorganisms and per cent removed by the gills. It is possible that differences in experimental techniques and in materials being filtered may adequately account for differing results.

Our experiments show a well defined pattern of particle removal when results are expressed in terms of per cent removal. Consistently and regardless of experimental conditions, oysters removed particles in the size range of 1.0 to 2.0 μ with less than half the efficiency of the larger particles. Efficiency increased with particle size up to 3.0–4.0 μ for natural particles and to 2.0–3.0 μ for kaolinite and then leveled off, with no evidence of a further change in efficiency. Minimum size of natural particles removed was between 0.8 and 0.9 μ, when 34.8% of the available particles were removed.

The increased efficiency of particle removal with an increase in particle size was not unique to flows of 1.01 l/min and 12 oysters. Doubling oyster number at 1.01 l/min had the effect of displacing the entire curve upward, 24% in the 1.0–2.0 μ range and from 34 to 42% in the larger size range. The inflection point on all curves, however, was still in the 3.0–4.0 μ range. A similar inflection point was also demonstrated when flows were reduced.

The percentage value at which the curves level off in our experiments is interpreted as representing the relation between the quantity of water filtered through the oysters' gills and the total volume of water flowing through the trough. Where the curve levels off at a removal of 60%, it is suggested that oysters were only pumping through their gills about 60% of the water flowing over them. This interpretation is supported by the fact that the curve was displaced upward when flows were decreased or oyster number increased without any changes in the nature of the curve itself. Furthermore, it was outlined earlier that oysters in our study were receiving 1.05 l/hr per gram of tissue. This is in excess of the 0.5 to 0.8 l/hr per gram of tissue pumping rates of adult oysters summarized by Jørgensen (1966). Leveling off of the curves, therefore, indicates that all particles larger than the size of which the inflection point occurs are being completely filtered out of the water pumped through the gills.

The constancy in location of the inflection point in the curves suggests that it represents the smallest particle size that oysters can retain with complete efficiency. Jørgensen (1966) stated that such a sharp size limit between retainable and non-retainable particles is an indication that it is particle size and therefore the porosity of a filter that is responsible for retention. He also stated that the critical size for particle retention in *Crassostrea virginica* corresponds to the distance between adjacent latero-frontal cilia on a filament, which according to Atkins (1938) varies between 1.5 and 3.7 μ. This range coincides with the range of particle sizes at which the inflection point appears on our curves. This correspondence reinforces the suggestion that the distance between adjacent latero-frontal cilia is the factor that determines the smallest particle size that can be completely retained by the oyster gill.

It is difficult to reconcile the preceding mechanism of removal with the observed quantity of material removed in the 0.8 to 3.0 μ range in our experiments. Several possibilities exist: (1) The Coulter Counter essentially measures volume of particles and results are expressed in terms of the diameter of spheres of equal volume. It would be illogical to assume that the smaller particles would be round since particles in the 0.8 to 3.0 μ size range might include lenticular clay particles, ovoid dinoflagellates, rod-shaped bacteria, *etc.* It is possible that the orientation of the long axis of these irregularly shaped particles as they strike the filtering mechanism results in retention of a fraction of their numbers. (2) Smaller particles may be trapped in with the larger ones, as suggested by Smith (1958), or adhere in some way to mucus on the gills. (3) Passage of particles through the interplicar spaces may stimulate blood to be pumped into the vascular tissue surrounding the ostia resulting in partial closure of these openings (Elsey, 1935). (4) The gill musculature may exert some regulation on the porosity of the gill filter by adjusting the width of the ostia (Jørgensen, 1966).

The mucous sheet theory of MacGinitie (1941) cannot be considered as a possible mechanism to explain the removal of particles in the 0.8 to 3.0 μ range in our studies. According to this theory, when the animal is feeding, a mucous sheet that covers the entire gill surface intercepts all particles from the water passing through the gills. In all individual experiments we conducted, efficiency of removal was lower in the 1.0 to 3.0 μ size range than at larger sizes. Existence of a mucous sheet should have resulted in complete retention of the smaller particles rather than in the partial retention observed. The plot for per cent removal vs. size would have then been a straight line showing equal efficiency of removal of particles of all sizes instead of the curve we obtained (Figs. 2 through 5).

Much controversy has evolved around the mucous sheet theory of MacGinitie (1941) as opposed to the straining function of the latero-frontal cilia. MacGinitie claimed that when the mucous sheet was absent from the gills of the four pelecypod species he studied, no feeding was taking place, in effect disregarding the involvement of ciliary mechanisms in particle retention.

Nelson (1960) objected to the mucous sheet theory on the basis that if a mucous net fine enough to trap bacteria were spread across the gills, it would be drawn down against the lateral cilia and into the ostia by the strong water currents pumped through the gills interfering with the function of both. Jørgensen (1966) stated that it is unlikely that the complicated sorting mechanisms of the bivalve gill should not play a part in their feeding process as would be the case if a mucous sheet was required. Owen (1966) expressed the same objection and added that such a continuous mucous sheet would be nearly impossible to maintain in the presence of the adjacent tracts of frontal cilia beating in opposite directions. Dral (1967) did not find any supporting evidence for existence of a mucous sheet in *Mytilus edulis* in heavy or dilute particle concentrations and pointed out that all observations indicated that the latero-frontal cilia constitute the filtration mechanism.

Jørgensen (1955, 1966) tried to conciliate the conflicting interpretations by suggesting that filtration by means of mucous sheets occurs in water that contains only small concentrations of suspended material and that in water with large particle concentrations, filtration is accomplished by ciliary mechanisms.

Our experiments covered a wide range of concentrations (Fig. 7). The curve for efficiency removal obtained in every experiment showed the same pattern described in the results. The presence of an inflection point in every instance, which has been given as an indication of the absence of a mucous sheet, suggests that particle retention was accomplished by a mechanism other than the mucous sheet at low as well as at high concentrations. Particle retention on the gills by latero-frontal cilia, however, is very probably enhanced by other responses such as muscular contraction of the gills and ostia, vascular enlargement of tissues around the ostia and secretion of mucus in a manner other than as a continuous sheet (Verwey, 1952; Owen, 1966).

The preceding discussion has considered particle filtration in terms of per cent removal since much of the previous work has been presented in this manner. From an ecological aspect, the data become more significant when presented in terms of volume. Although per cent removal was lower between 1.0 and 3.0 μ for natural particles and 1.0 and 2.0 μ for kaolinite than at larger sizes, volumes removed in these size ranges constituted the largest single fraction within the total size range investigated. This resulted from the smaller particles between 1.0 and 3.0 μ being many times more numerous than those of larger size (Table I).

The oysters filtered relatively large quantities of solids in the 0.8 to 3.0 μ size range. No effort was made in this study to differentiate between the fraction of the particles filtered out and ingested and that of particles filtered out but rejected as pseudofaeces. However, data collected in concurrent studies suggested that particles in this size range are the major component of solids ingested by oysters. Approximately 95% of the particles counted microscopically in dispersed samples of oyster faeces were smaller than 3 μ (Haven and Morales-Alamo, 1966a). Eighty to ninety per cent by volume of the particles in similar samples were smaller than 4 μ as determined with the Coulter Counter (Haven and Morales-Alamo, 1968).

These findings suggest that particles in this size range suspended in the water may play an important role in the nutrition of oysters and other lamellibranchs. A review of much of the existing information on size of particles suspended in coastal waters is given by Jørgensen (1966). He concludes that in respect to the phytoplankton which are of importance in the nutrition of lamellibranchs, the nanno- and ultraplankton (less than 5.0 μ) mostly constitute the larger and often even the dominant part of the phytoplankton. Included in the size class are the marine bacteria which may range in size from less than 1 μ up to about 2.5 μ.

Organic detritus has also been suggested as being an important food source for filter-feeding lamellibranchs (Verwey, 1952). Mullin (1965a) found that the 1–10 μ fraction of the particulate matter from 20 stations in the western Indian Ocean constituted on the average 58% of the total particulate organic carbon measured. Subsequently, Mullin (1965b) stated that phytoplankton carbon accounted for less than 20% of the total particulate carbon, making carbon in the form of detritus or heterotrophic organisms considerably more important than was suggested in his earlier report. Newell (1965) suggested that much of the food supply of filter feeders may be provided by microörganisms attached to fine size suspended organic and mineral particulate matter.

Removal of fine particles in the 1 to 10 μ range by oysters and possibly by other filter feeders may also be an important factor in sedimentary processes affecting the physical and chemical characteristics of bottom substrates and the benthic communities they support (Haven and Morales-Alamo, 1966a, 1966b, 1968). In a similar manner, radionuclides adsorbed onto clay mineral particles or incorporated into bacterial and algal cells as a result of accidental contamination of an estuary may be removed from the water by the filtration activity of filter feeders and deposited on the bottom where their accumulation may become a hazard.

Appreciation is expressed to Mr. John Norcross, Dr. Joseph Loesch, and Mr. Frank Wojcik of the Virginia Institute of Marine Science for assistance in statistical analysis and in preparation of computer programs.

SUMMARY

1. Particle filtration by the oyster *Crassostrea virginica* was studied in the 1.0 to 12.0 μ size range in relation to naturally occurring particles and to kaolinite suspensions in filtered river water.

2. Oysters were held in troughs of flowing water under conditions similar to their natural environment. Particle number and volumes entering and leaving the troughs were enumerated using a Coulter electronic particle counter. Particle diameter was expressed as that of a sphere having a volume equal to the particle.

3. Results of the study were expressed as per cent of total particles removed in various size increments or in volumes removed over the same size range.

4. Oysters filtered naturally occurring particles in the 1.0 to 3.0 μ range with about one-third the efficiency as larger particles. Above 3.0 μ there was no change in efficiency with increasing particle size. For kaolinite particles, oysters removed particles in the 1.0 to 2.0 μ range with about half the efficiency as larger particles. Above this size there was no change.

5. When results are expressed in terms of per cent removal, the importance of the small sized particles is minimized. In terms of volume, particles in the 1.0 to 3.0 μ range constitute the largest single size fraction over the 1.0 to 12.0 μ range.

6. The consistent presence of an inflection point at a particle size around 2.0–3.0 μ in the curve for per cent removed *vs.* particle size is an indication that the distance between adjacent latero-frontal cilia is the factor that determines the smallest particle size that can be completely retained by the oyster gill.

7. Presence of the inflection point is also interpreted as negating the existence of a mucous sheet over the gills, as suggested by MacGinitie (1941).

8. Particles in the 1.0 to 3.0 μ range may play an important role in the nutrition of oysters and other lamellibranchs. Their removal by these organisms may also be an important factor in sedimentary processes.

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Reference: *Biol. Bull.*, **139**: 265–276. (October, 1970)

CHANGES IN MICROTUBULES OF CILIA AND FLAGELLA FOLLOWING NEGATIVE STAINING WITH PHOSPHOTUNGSTIC ACID[1]

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Phosphotungstic acid (PTA) is a reagent commonly used for the negative staining of cilia, flagella and certain other structures (see, for example, Burton, 1970). In addition to its more or less passive role in outlining and filling such structures, however, there are also macerating and digestive effects on microtubules, and very little direct attention has been paid in the literature to these. The protein-precipitating action of PTA at pH 5 is well known, but to my knowledge its lysing action at pH 6.8 has never adequately been documented. The present report is an attempt to do so, and to give some data on the selective nature of such effects among various types of microtubules.

MATERIALS AND METHODS

Both freshwater and marine turbellarian flatworms were used, the former collected locally from University Lake and from Stone Mountain, Georgia, and the latter furnished by the Supply Department of the Marine Biological Laboratory at Woods Hole, Massachusetts. They included the following: *Mesostoma georgianum, Microdalyellia* sp. and *Macrostomum* sp., all freshwater rhabdocoels; the alloecoel *Monoophorum* sp. (marine); and a marine polyclad, *Stylochus zebra. Prostoma rubrum,* a freshwater rhynchocoel, was also studied.

Maceration and negative staining were done with a 1% aqueous solution of phosphotungstic acid brought to a pH of 6.8 by the addition of 1 *N* KOH or NaOH. A trace of bovine serum albumin was added just before use. The larger animals were cut up with sharp needles and the pieces immediately put into PTA; smaller forms, such as *Macrostomum* and *Microdalyellia*, were dropped intact into the reagent. At appropriate intervals, samples were removed to Formvar-carbon-coated 200-mesh copper grids and treatment for all was continued at room temperature for periods varying from 2 to 10 min, after which the PTA was rapidly withdrawn with filter paper and the preparation allowed to dry. Micrographs were made with the Zeiss 9A and 9S electron microscopes.

Microtubules both of cilia (*Prostoma, Microdalyellia, Monoophorum, Mesostoma* and *Stylochus*) and of flagella (spermatozoa of *Mesostoma*) were studied, as well as the ciliary rootlets of *Macrostomum*.

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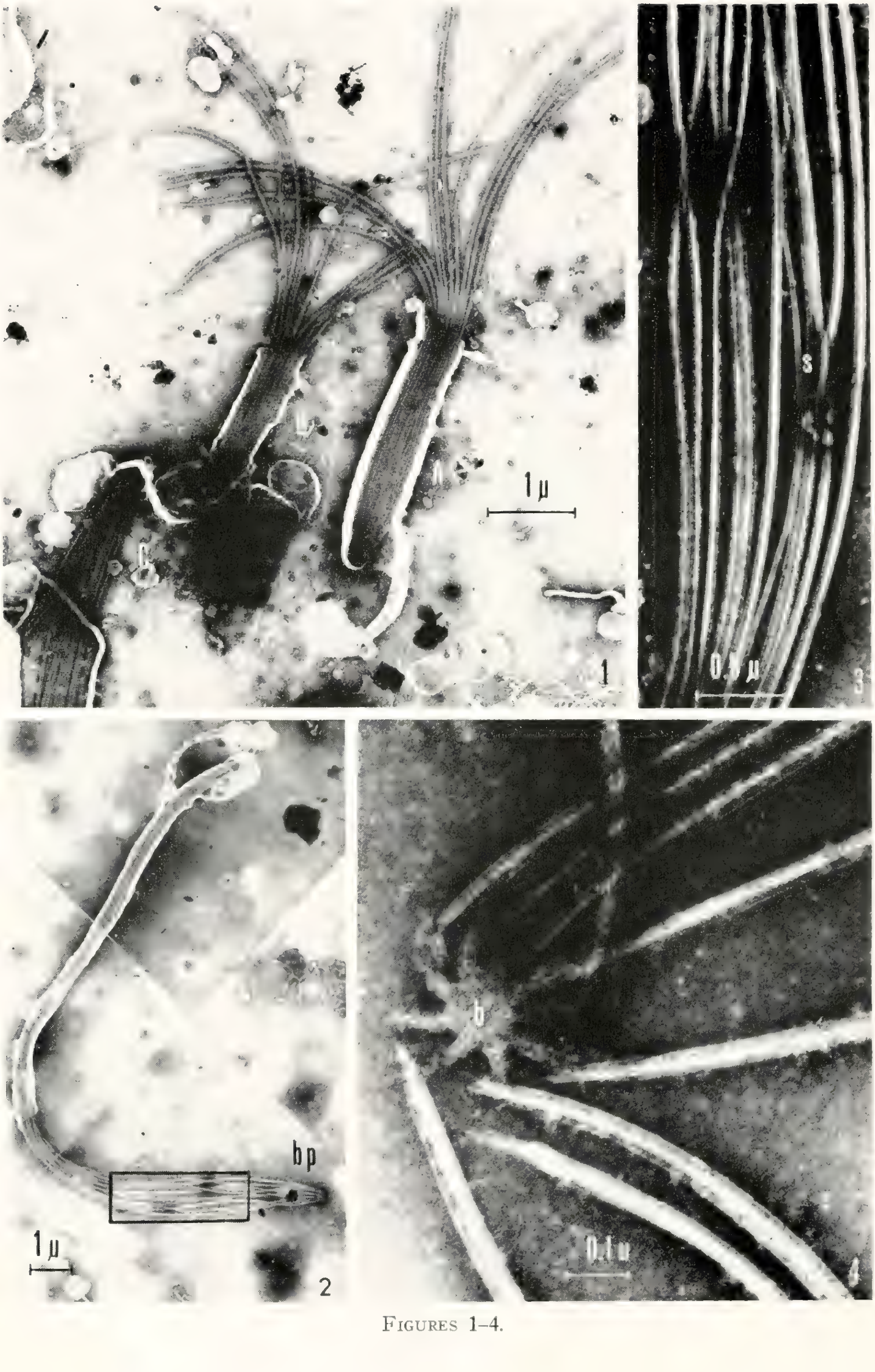
[1] Aided by a grant from the National Institutes of Health, GM 15311.


1 μ
1
s
0.1 μ
3
bp
1 μ
2
b
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4

Figures 1–4.

OBSERVATIONS

Disappearance of one or both of the two central singlets in PTA-treated cilia and flagella, in the absence of noticeable effects on the doublets, is one of the very few predictable findings. This is well illustrated in Figure 1, showing 3 cilia, in varying stages of maceration but in close physical juxtaposition to one another on the same grid square. In cilium A (right) all 9 doublets and the 2 central singlets are present; the boundary of the binding matrix (arrow) is considerably farther distal along the axoneme than it is in cilium B, and the microtubules are longer. Furthermore, one of the 2 central singlets in B is entirely absent and the other is present for only a short distance. There is some evidence in higher-magnification micrographs that at least one of the doublets in B has likewise begun to disintegrate. In cilium C (lower left corner) the binding matrix has almost entirely disappeared and only the 9 doublets remain.

This variability in macerating action is also apparent among different grid squares of the same preparation, as shown by the cilia in Figures 2–5, all of which were on one grid. An entire cilium is shown in the montage of adjacent micrographs in Figure 2; here, very little maceration has occurred at the distal end of the cilium (upper), while at the basal region the binding matrix is absent and the 9 doublets and 2 singlets are well separated (Fig. 3), with very few, if any, signs of degeneration. The star-shaped basal plate in Figure 4 shows evidence of a considerable degree of degeneration; the 9 doublets are all present but most are broken off from the plate. The connections of the doublets to the basal plate appear to be effected by tapering processes.

The microtubules and basal plate of the cilium in Figure 5 are in a rather advanced stage of deterioration. Only portions of the 2 singlets (**s**) are still present and their protofibrils are conspicuous. There is considerable variation along the lengths of the 9 doublets. Both subtubules are mostly intact in the two doublets marked with white arrows; all the others have at least portions of one member absent. There appears to be a general tendency for the degradation process to begin proximally and proceed to completion distally, but sometimes this progression is interrupted, as at the breaks indicated by **b**. Behnke and Forer (1967) and Stephens (1970) have demonstrated that the B-member of the doublet is more thermolabile than the A-. Since the surviving members of

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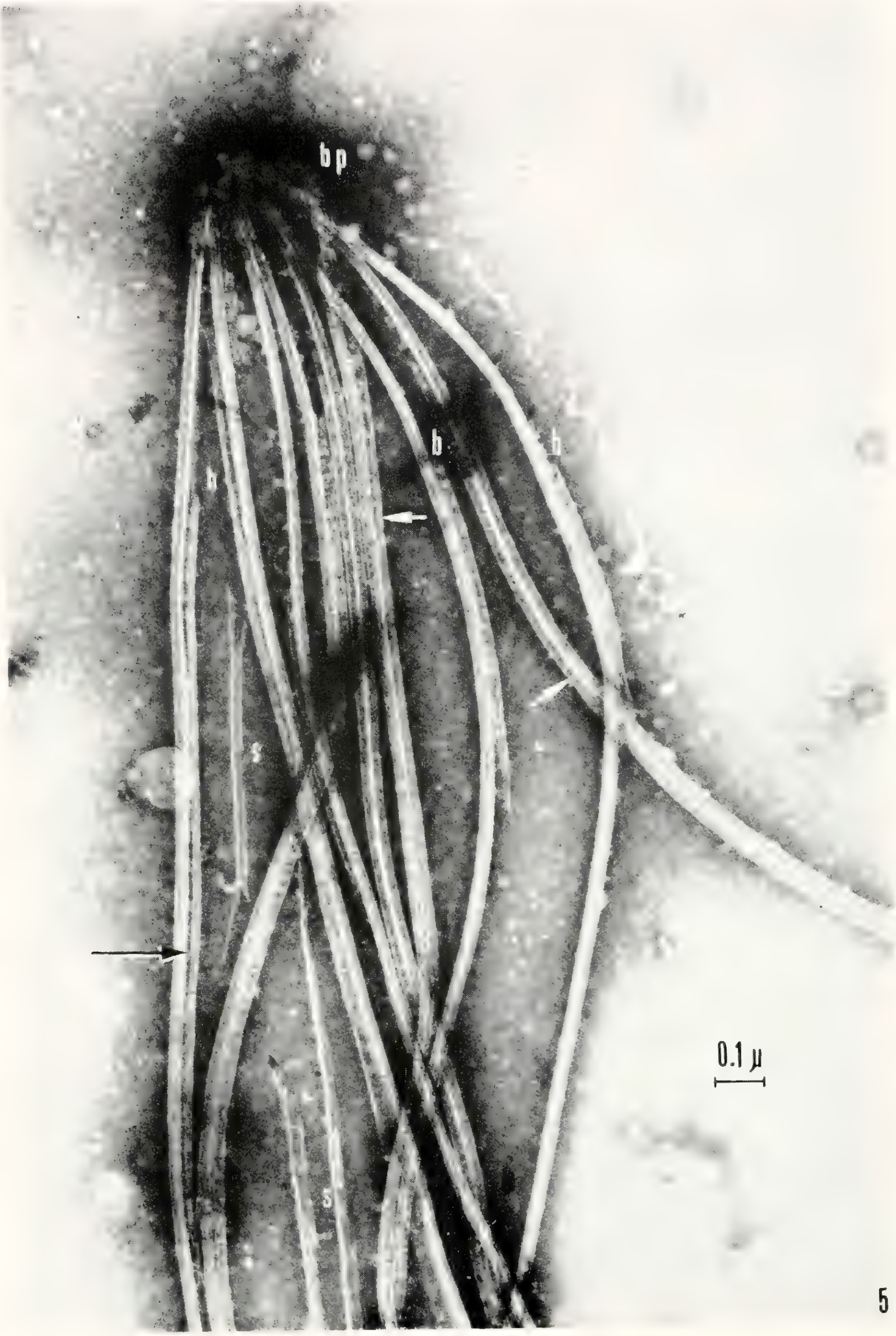
FIGURE 1. Three cilia in varying stages of maceration, from the same grid square. In cilium A, all 9 doublets, the 2 central singlets, the matrix and membrane (the position of which is designated by the arrow) still are present. In cilium B, all 9 doublets are present but only 1 of the 2 singlets, and that for only a short distance. Nearly all the binding material has disappeared from cilium C, as well as both central singlets, leaving just the 9 doublets; *Prostoma rubrum;* 4-min treatment.

FIGURE 2. A montage of adjacent micrographs of a cilium; the basal plate end (**bp**) is well macerated and the 9 doublets and 2 central singlets are well defined here, but the membrane and matrix, and possibly some coagulated mucus, are still present along the distal two-thirds of the axoneme; *Microdalyellia;* 10-min treatment.

FIGURE 3. Enlargement of the area indicated on Figure 2. The two singlets lie on either side of the letter **s**. Scale designation should be 0.4 micron instead of 0.5.

FIGURE 4. End-on view of a ciliary basal plate (**b**); only the 9 doublets are present, and of these, only 4 have even a trace of remaining attachment to the plate; *Microdalyellia;* 10-min treatment.


bp
b
b
b
s
s
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5

FIGURE 5.

each doublet in Figure 5 have complete walls, and since the A-member is known to be the complete one (Stephens, 1970), it seems safe to surmise that we have another example of the greater sensitivity of the B-tubule to chemical and physical agents (in this case, PTA). Besides bringing about a complete disappearance of the B-subtubule, the macerating action of PTA may result in localized loss or separation of some of the component protofibrils for short distances (black arrow), so that a longitudinal split is present.

Figures 6–9 are micrographs of successive levels along a cilium *ca.* 14 microns long; Figures 6 and 7 are immediately adjacent to one another, as are Figures 8 and 9. Here the macerating action of PTA after a 10-min treatment differs in another way from the effects discussed above. The two central singlets (arrows in Figure 6) have not yet disappeared, but have a very frayed appearance, with conspicuous protofibrils. This is also the case for the doublets along their entire lengths, although it is less striking than in the singlets. There is little evidence in the doublets of the white line which, in optimally "stained" PTA-treated material, marks the common wall between the A- and B-subtubules of a doublet. Quite frequent breaks also occur along the doublets, as well as bends (Fig. 7).

An even more drastic macerating action of PTA is seen in the ciliary microtubules shown in Figure 10. Degradation here is so advanced that one cannot state with certainty which are doublet and which singlet microtubules; only 8 are present of the expected total of 11 and there was no evidence nearby on the grid square of the presence of the others. The existence of a helical arrangement of subunits is suggested at the point marked by the arrow, and elsewhere as well.

A difference in the effects of PTA on flagellar axonemes and on the cortical singlets of the spermatozoon of *Mesostoma* (Henley, Costello, Thomas and Newton, 1969) is shown in Figure 11. Here, after a 3-min treatment, the flagellar axoneme (**fa**) is still almost unaffected by PTA, while the cortical singlets (**cs**) are thrown into a striking helical configuration. It is not apparent in this low power micrograph, but in addition to the gross PTA-stimulated spiralling, the cortical singlets have a marked helical pattern in their walls, when viewed at higher magnifications. This is very similar to the configuration demonstrated in cortical singlets of the spermatozoon of *Stylochus* by Thomas (1970). We have thus far found no evidence of comparable helical configurations in cilia on the same grids as the coiled cortical singlets of spermatozoa, suggesting that here is another differential effect of PTA on different types of microtubules. The spiralling of the entire complement of cortical singlets shown here can readily be related to an abrupt coiling of certain spermatozoa (including that of *Mesostoma*) observed by phase contrast microscopy during treatment of living spermatozoa with PTA. This coiling may persist for a period of 20 min or more of continued treatment, after which it disappears and the spermatozoa again assume a more straightened form. There is thus the possibility that at a certain stage of its action, PTA stimulates the microtubule subunits to undergo some of the changes normally associated

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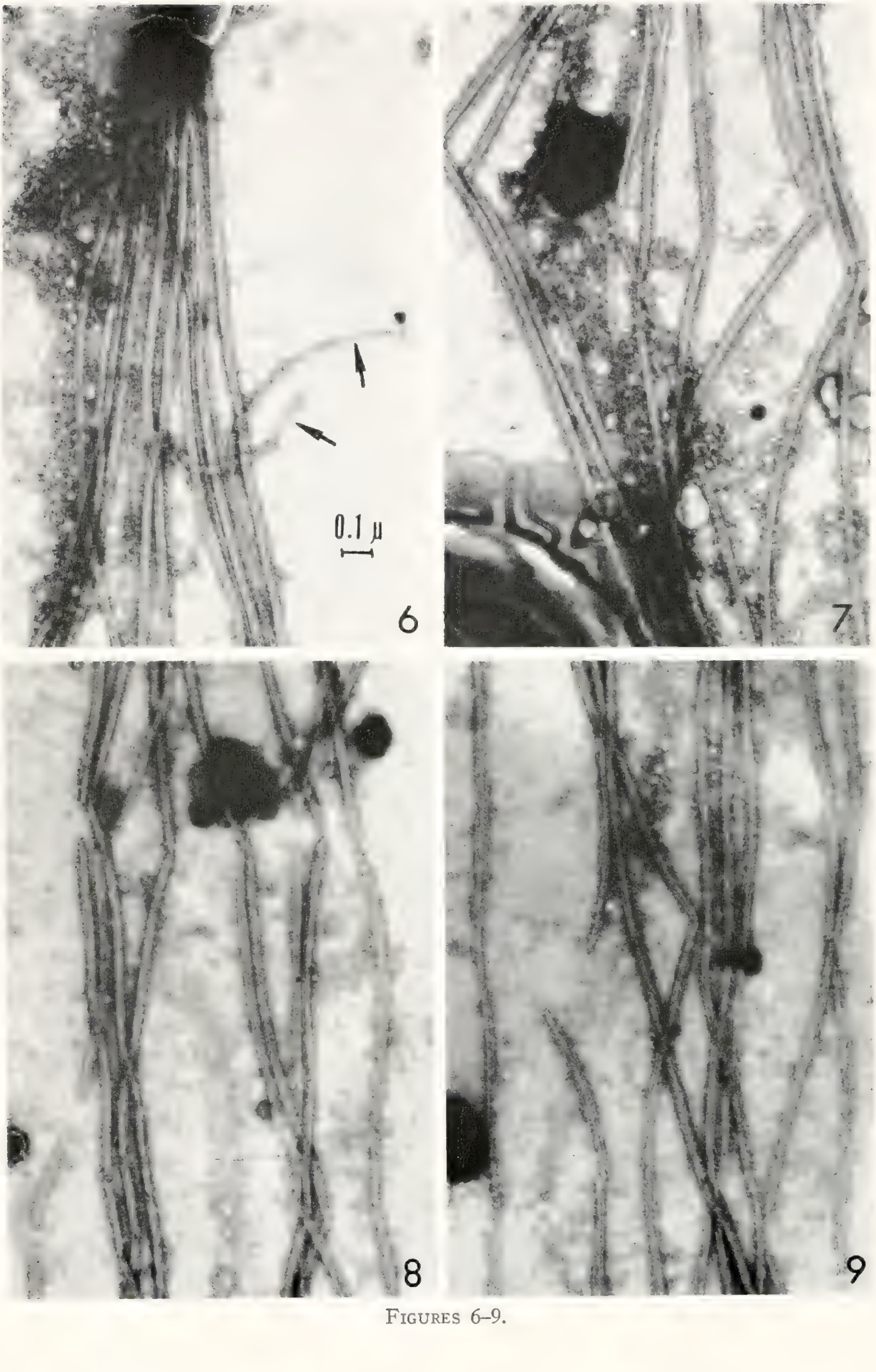
FIGURE 5. Considerable maceration of the microtubules has occurred in this cilium. The 9 doublets and fragments of the singlets (**s**) are present, but only the 2 doublets designated with white arrows are intact or nearly so. One member of the doublets (probably the B-subtubule) has completely disappeared at the regions marked **b**, and a longitudinal separation between the two is apparent at the black arrow, **bp**, basal plate; *Microdalyellia;* 10-min treatment.



FIGURES 6–9.

with the production of motility. Continued action of the PTA macerates the microtubules more, and their "contractility" is lost. There is as yet no way of knowing whether PTA induces spiralling of the singlets, or whether the coiling results from release of an inherent tendency towards spiralling which is facilitated by PTA's dissolution of the spermatozoon's plasma membrane.

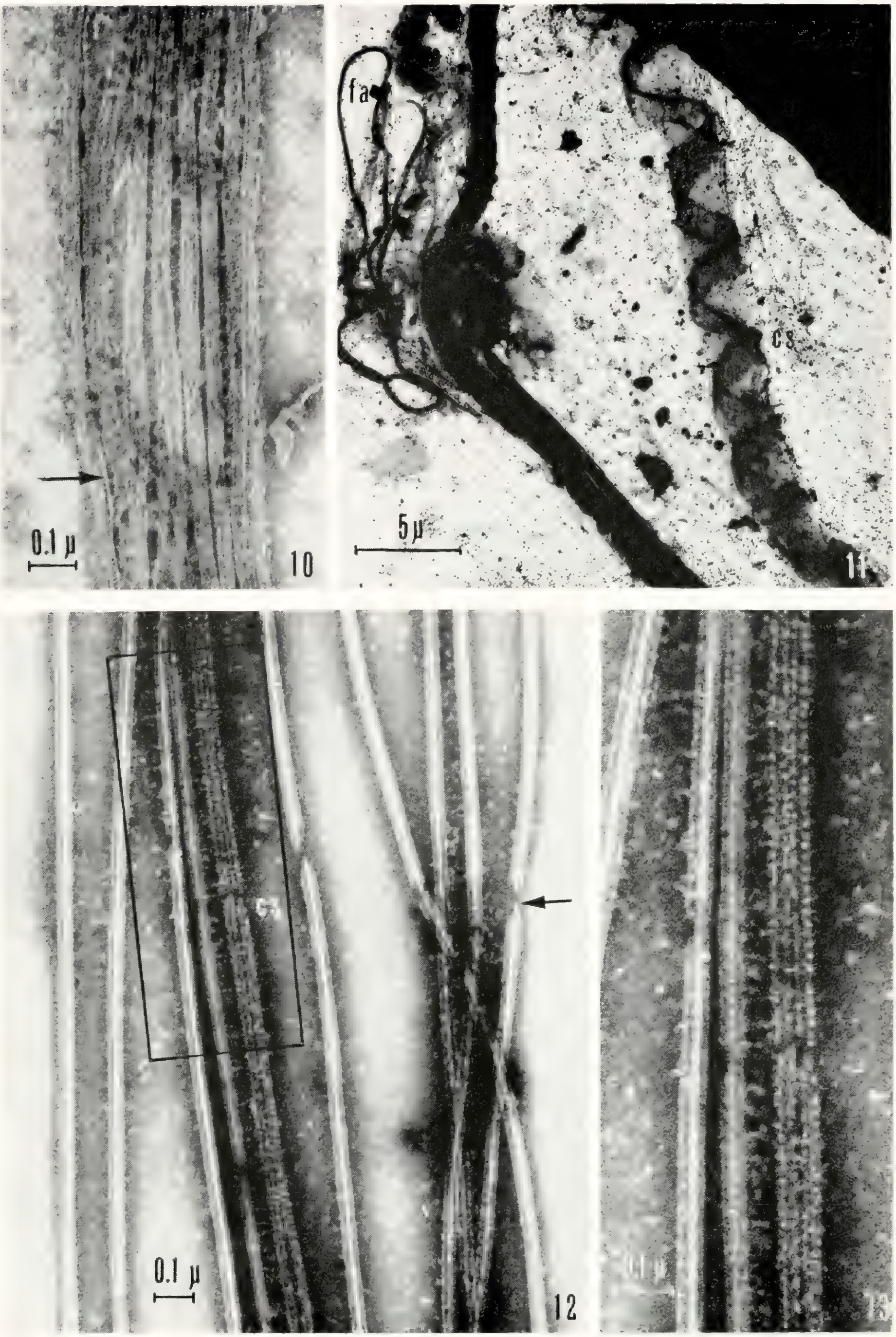
The pH of the PTA solution appears to be of rather critical importance, for even very short treatments at pH 8.3 (Figs. 12 and 13) result in changes in the 2 central singlets of cilia which are quite unlike those observed after any other treatment thus far tested. The singlets become semi-fused together along much of their lengths, and their component protofibrils have a beaded appearance, similar to that described by Behnke and Forer (1967) for central singlets after negative staining at pH 7. Some breaks are also apparent in the doublets, but both members reappear beyond the breaks. The common walls between the subtubules can be seen to persist in the absence of members of the doublet at the point marked by an arrow.

One or more striated ciliary rootlets per cilium are present in sections of all the forms studied here, but are rarely encountered in PTA-treated material. This suggests that they are very sensitive to the digesting action of the reagent at the concentration and pH used, and that they therefore disappear rapidly. Figure 14 shows two rootlets which did survive the treatment; there is a conspicuous major periodicity of *ca.* 790 Å and evidence of a longitudinal fibrous substructure as well. The montage of adjacent micrographs in Figure 15 shows a rootlet in which the process of degeneration is quite far advanced at one end, while the other end (at the top of the picture) still retains the clear 790 Å repeating pattern. The longitudinal fibrous substructure is clear in the lower region, but the periodicity here is obscured. Dorey (1965) observed a 650–700 Å repeating pattern in negatively stained ciliary rootlets from a number of acoels; he used 0.05% PTA at pH 6.2 (as opposed to 1% at pH 6.8 used in the present work) and treated for periods of 5–10 min. The differences in technique may account for the disparity in periodicity, or there may be species differences. Further work to elucidate this point is in progress.

DISCUSSION

The impressive variety of degenerative changes in microtubules and associated structures, and the general unpredictability of effects exerted by PTA lead one to the belief that perhaps a great deal of caution should be exercised in the interpretation of electron micrographs of negatively stained material. It is not clear, for example, exactly which of the changes described by such workers as Behnke and Forer (1967) and Burton (1968) were due to experimental manipulations (using such agents as colchicine, temperature, pepsin, *etc.*) and which to the action of PTA itself.

FIGURES 6–9. Micrographs at successive levels along one cilium, showing conspicuous protofibrils and many breaks. The complete series of 7 micrographs comprising this group reveals that every doublet has at least one break along its length, and several have more than one. The arrows in Figure 6 designate the disintegrating central singlets. Note the rather sharp bends in the microtubules of Figures 7 and 9. Figures 6 and 7 are immediately adjacent to one another, as are Figures 8 and 9; *Monoophorum;* 10-min treatment.


0.1 μ
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Figures 10-13.

Behnke and Forer (1967) found that the central singlets of the 9 + 2 configuration were more likely to be absent in PTA-treated material, confirming the results reported here and by many other workers (see their Table 3). Grimstone and Klug (1966) showed that the central singlets in flagella of a number of protozoa from *Cryptocercus* and *Zootermopsis* only rarely survived even very short treatments (15 sec) with 1% PTA, pH 7.0. If the central singlets were present, however, these were observed to be uncollapsed, in contrast to the doublets which readily collapsed or frayed.

Another type of central element in axonemes has been studied by Burton (1968) and Thomas (1970), among others, in spermatozoa of the lungfluke *Haematoloechus* and the polyclad turbellarian *Stylochus,* respectively. They both found the complex central core of the "9 + 1" pattern to be more resistant than either the doublets or the cortical singlets to a variety of experimental treatments, followed by negative staining with PTA. These data of Burton and of Thomas for spermatozoa are in interesting contrast to those presented here for cilia, which implicate the central elements as the least resistant to the action of PTA. However, the relationship of central singlets in the 9 + 2 pattern to the comparatively massive core in the "9 + 1" pattern is obscure and probably complex. It may be, also, that there is an inherent difference in the properties of microtubules in cilia and in spermatozoa of the same form. Paired "9 + 1" axonemes, as well as cortical singlets, are found in spermatozoa of 3 (*Mesostoma, Microdalyellia* and *Stylochus*) of the 6 forms included in the present report. The spermatozoon of *Macrostomum* has cortical singlets only, with no axonemes (Henley, unpublished data). In the other two (*Monoophorum* and *Prostoma*) the axonemal pattern of spermatozoa has not yet been studied, but on the basis of information we have gathered from 8 other free-living flatworms, 1 other rhynchocoel and 3 annelids, it is possible to state that there is no set relationship between the pattern of microtubules in spermatozoa and in cilia of the same form. In addition, the fact that the cortical singlets of the spermatozoon of *Mesostoma* (and of *Microdalyellia* and *Stylochus* as well) react differently to PTA than do the microtubules of the flagella suggests yet another source of variability.

Differences in susceptibility of microtubules to PTA (and to other treatments as well) such as those discussed above may very well involve subtle differences in the biochemical composition of these structures in the various groups, despite their

FIGURE 10. Ciliary microtubules so completely macerated that one cannot determine which (if any) are singlets and which doublets. Arrow designates helical particulate substructure. Three of the normal total complement of microtubules are present; *Mesostoma;* 6-min treatment.

FIGURE 11. Cortical singlet microtubules (**cs**) of a spermatozoon (at right) are thrown into a conspicuous helical arrangement by PTA treatment, while the microtubules of the flagellar axoneme (**fa**) are not. The thick solid black structure in the center is probably an unmacerated spermatozoon; *Mesostoma;* 4-min treatment.

FIGURE 12. This ciliary axoneme was treated with PTA at pH 8.3, rather than the usual 6.8. All 9 doublets are present, as well as the 2 central singlets (**cs**), which appear to be almost fused together and which have a striking beaded appearance. The breaks in the doublets are somewhat sharper than those usually seen after treatment at the lower pH; *Stylochus;* 2-min treatment.

FIGURE 13. Enlargement of the area indicated by the rectangle in Figure 12. (Micrograph by Mary Beth Thomas).

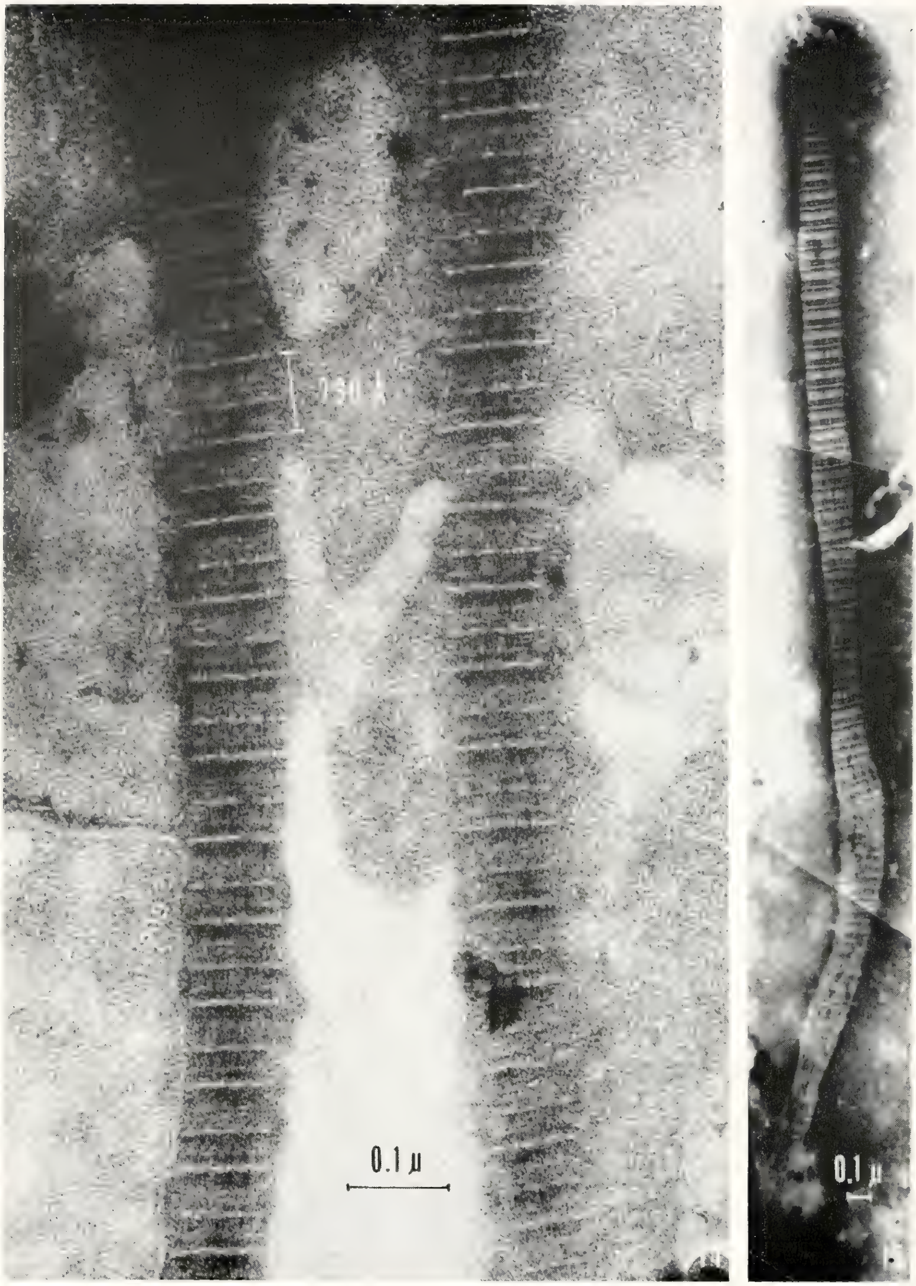

FIGURE 14. Two ciliary rootlets, negatively stained with PTA, showing a major repeating pattern of *ca.* 790 Å. Note the longitudinal fibrous substructure; *Macrostomum;* 4-min treatment.

apparent morphological resemblances. Evidence for this is offered by the recent work of Behnke (1970), who studied the comparative sensitivity of microtubules in disk-shaped blood cells of the frog, chick, rat and man, to cold, Colcemid, *N*-ethylmaleimide and alkaloids of *Vinca*. He found very marked species differences in such susceptibility. In our material, there are undoubtedly great variations in such features as the toughness of the pellicle, the presence or absence of mucus, *etc.*, which could affect the action of PTA and the required duration of treatment for adequate negative staining. Also, it is our experience that marine forms are considerably less sensitive to the macerating action of PTA than freshwater ones, perhaps because of the presence in seawater of divalent ions, which appear to inhibit the action of PTA. In this connection, Roth and Shigenaka (1970) have recently shown that microtubules of the heliozoan axopod respond to treatment with cupric ion very much as they do to colchicine. They were also found to be very susceptible to degradation in the presence of nickel ion. The concentrations of both these cations in sea water are low (copper, 0.001–0.09 mg/l, nickel, 0.0001 mg/l, according to the *Handbook of Chemistry and Physics,* 1970, page F-145), but they could well be involved in the observed variability in the action of PTA.

It may be pertinent to point out here that while the precise formula of phosphotungstic acid is not entirely clear (due to the amount of included water of crystallization), its molecular weight is high. Values of 3312.5 and 3132.4 are given (*Handbook of Chemistry and Physics,* 1970), for molecules containing 24 and 14 units of water, respectively. Therefore, a 1% solution is only about 0.003 *M*. Since the spermatozoa of many marine forms are osmotically adjusted to survive for a considerable period of time in sea water of molarity equal to that of a 0.55 *M* NaCl solution, the 1% PTA is decidedly hypotonic. Some of the gross changes in living sperm subjected to PTA treatment may be due to this factor.

One of the most unpredictable aspects of negative staining is the correct duration of treatment with PTA, to achieve optimal "staining" of the microtubules without overmaceration. This seems to be correlated, to some extent at least, with the species differences already alluded to; for each new form we study, the correct duration of treatment is determined empirically and has been found to range from a few seconds to 30 min or more.

Burton (1966) briefly discusses the variability in action of PTA, in material on consecutive grids treated in the "same" way, and points out (page 404) that studying "negatively stained material forces one to make value judgments." These value judgments involve such factors as taking into account the varying degrees of susceptibility of the different types of microtubules and other components of cilia and flagella, to the degenerative effects of PTA. That this is indeed the case is abundantly borne out by the results reported here, in negatively stained microtubules on the *same* grid and, in fact, on the same grid square.

FIGURE 15. Montage of three adjacent micrographs, showing progressive maceration of a negatively stained ciliary rootlet. The basal body end (top) is intact, but there is progressive disintegration, ending in a completely disorganized mass at the distalmost end; *Macrosotomum;* 10-min treatment.

I am indebted to Miss Mary Beth Thomas for permission to use her unpublished micrographs (Figs. 12 and 13), and to Miss Thomas and Dr. Donald P. Costello for valuable assistance in preparation of the illustrations.

SUMMARY

1. Variability in the macerating action of 1% aqueous phosphotungstic acid, pH 6.8, is exemplified in microtubules of flatworm and rhynchocoel cilia, from one grid square to another of the same preparation and within a single grid square. The central singlets appear to be the most susceptible and are often completely absent, even in cases where the binding matrix is still present around the doublets. Maceration usually, but not always, begins at the distal tip of a cilium and proceeds towards the basal plate; it is evident along the lengths of doublets as partial or complete loss of one subtubule, as breaks and bends, and as fraying into the component protofibrils, with disappearance of the white line marking the wall between the subtubules.

2. Cortical singlet microtubules of a spermatozoon were thrown into a helical configuration by the action of PTA, while the flagellar microtubules of the same spermatozoon were unaffected.

3. After treatment with 1% PTA at pH 8.3, the central singlets of cilia (but not the doublets) were semi-fused along most of their lengths, and had a beaded appearance; there were breaks in the doublets but otherwise they appeared to be unaffected.

4. Ciliary rootlets were rarely seen, but when present had a clear 790 Å major repeating pattern and a longitudinal fibrous substructure.

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THE ORIGIN, DISTRIBUTION AND FATE OF THE MOLTING FLUID PROTEINS OF THE CECROPIA SILKWORM

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Molting in insects is accompanied by the production of a molting fluid which fills the exuvial space between the new and old cuticles and digests the inner layers of the old cuticle. Most of the products of digestion are resorbed. This process has been studied in detail in the Cecropia silkworm by Passonneau and Williams (1953). During the pupal-adult transformation of the Cecropia silkworm, molting fluid appears in the exuvial space as soon as the epidermis retracts from the old pupal cuticle. This fluid persists for the first 19 days of the 21 days of pharate adult life. For the first two-thirds of this period the molting fluid is gel-like and has no obvious effect on the overlying pupal cuticle. However, on about the fourteenth day of pharate adult development, the molting gel of the pharate adult liquifies and begins to hydrolyze the proteins and chitin of the pupal endocuticle. By the 20th day the endocuticle has disappeared, leaving a thin crisp exocuticle and epicuticle. On the 19th day, molting fluid begins to be absorbed and, by the 20th day, most of it is gone from the exuvial space. On the 21st day, the insect emerges.

It is generally believed that the epidermal cells secrete the molting fluid. Whether they synthesize all of it, or secrete into it some components that were synthesized elsewhere, is not known. The literature contains only scanty references to the protein components of molting fluid. Passonneau and Williams (1953) showed that the early molting gel of Cecropia contains about 5 per cent protein. They also demonstrated proteolytic and chitinolytic activity in the molting fluid. Chen and Levenbook (1966) examined the molting fluid and blood of the blowfly, *Phormia regina*, by disc electrophoresis and reported that both fluids contained several proteins with similar R_f values. Their electrophoretic techniques did not permit them to determine whether any proteins of the molting fluid were related to those of the blood, or were products of cuticular digestion.

The mechanism of absorption of molting fluid prior to ecdysis is also unknown. It is generally believed that the ability to absorb molting fluid is a generalized property of the integument and that absorption takes place through the *general surface* of the new cuticle (Passonneau and Williams, 1953; Wigglesworth, 1933, 1948, 1965). However, it is not known whether specific regions of the integument are specialized as sites of absorption. Also, although it has been shown that small molecules are absorbed (*cf.* review by Noble-Nesbitt, 1967), nothing is known of the possible absorption of macromolecules.

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This report examines the proteins of molting fluid, epidermis and blood during the pupal-adult transformation of the Cecropia silkworm, by immunological and electrophoretic techniques. It considers the following questions:

1. Which proteins (if any) are found in both molting fluid and blood, and where are these proteins made?
2. To what degree do native proteins (from blood and molting fluid) and introduced foreign proteins (from rabbit serum) move from molting fluid to blood and vice versa during adult development. How much macromolecular "traffic" is there between the blood and the fluid in the exuvial space?
3. Are macromolecules absorbed when molting fluid is absorbed just prior to ecdysis?
4. Is molting fluid absorbed by the general surface of the newly formed integument or does absorption occur more readily at certain sites?

MATERIALS AND METHODS

Experimental animals

Larvae, pupae and pharate adults of the Cecropia silkworm, *Hyalophora cecropia,* were used in most experiments. Males and females were used interchangeably since there appeared to be no sex-limited differences in the phenomena we examined. In a few experimnts larvae of *Samia cynthia ricini* and *Galleria mellonella* were used. Methods of rearing, storing, handling and staging these insects have been described elsewhere (Krishnakumaran, Berry, Oberlander and Schneiderman, 1967; Schneiderman and Williams, 1954; Telfer and Rutberg, 1960). All animals were maintained at 25° C.

Reagents

Chemicals used for disc electrophoresis and immunoelectrophoresis were routine commercial preparations. Serum proteins came from laboratory rabbits. Purified rabbit gamma globulins (7S and 19S) were obtained by fractionation on DEAE cellulose columns and were lyophilized. All of the insect tissues were washed in 0.85% NaCl.

Collection of fluid in tissue samples from developing adults

Molting fluid, blood and tissue samples were taken from Cecropia silkworms of specific ages. Phenylthiourea (PTU) was added to all wounds and to all samples of tissue and fluids. In most experiments the fluids or tissue homogenates were centrifuged at 15,000 rpm in a Beckman "microfuge" for four minutes and stored at −20° C until used.

Molting fluid: A small piece of cuticle was excised from the tip of the abdomen, and approximately 200 μl of molting fluid was collected in a capillary. During this process it was necessary to avoid touching the delicate epidermis with the capillary. Otherwise, tissue damage occurred and caused blood and molting fluid to mix. Molting fluid collected in the way described was not contaminated by blood (see Results). Occasionally, molting fluid was collected from antennae or wings for special purposes, but this fluid was often contaminated by blood.

Blood: Blood was collected from larvae, diapausing pupae and adults by means of fine glass needles inserted into the heart. To avoid contaminating the blood of pharate adults with molting fluid, special procedures were employed. In early pharate adults, an incision was made through the pupal cuticle along the dorsal midline of the abdomen. At this time, the epidermis is still closely pressed against the cuticle above the heart, and blood was collected from the heart without contamination by molting fluid. In late pharate adults, the pupal cuticle was removed, the animal washed in saline and blotted to avoid contamination by molting fluid. A sharp capillary was then inserted through the cuticle of the pharate adult into the heart to collect blood.

Epidermis: The developing wings and antennae are a convenient source of epidermis. However, the blood present in these appendages made it impossible for us to obtain epidermis free of blood, even though the tissues were cut into small fragments and washed repeatedly. In contrast, the abdominal epidermis could be isolated and freed of blood by careful dissection, fragmentation and repeated washing in ice cold saline. The fragments were blotted to remove excess saline, homogenized, centrifuged and the clear supernatant used for further analysis. Abdominal epidermis prepared in this way had no detectable amounts of major blood proteins.

Disc electrophoresis

For disc electrophoresis the procedure of Davis (1964) was followed, using *tris*-glycine buffer (pH 8.6) and applying 3 MA per gel for 30 minutes at 25° C. The procedure was modified slightly to meet our special needs (*cf.* Patel and Schneiderman, 1969 for details). Gels of several pore sizes were tested. A 4.3% gel provided the best resolution for blood, molting fluid and epidermal proteins and was employed in most experiments. Gels were stained after electrophoresis with Buffalo Black (=Naphthol Blue Black, Allied Chemicals) and were destained and preserved in 10% acetic acid.

Immunological methods

Antisera to blood proteins of diapausing pupae and adults of male and female Cecropia silkworms were prepared in rabbits. The insect blood was emulsified in Freund's adjuvant and injected subcutaneously into rabbits in four to six sites along the vertebral column. Two weeks later boosters were injected into the same sites. The antisera were deepfrozen at −20° C until used. These antisera could detect up to twelve different Cecropia blood antigens in immunoelectrophoresis experiments. Sheep antisera against total rabbit serum proteins and against rabbit gamma globulins were prepared by conventional methods.

The immunoelectrophoretic procedures of Grabar and Williams (1955) and the modified technique of Scheidegger (1955) were used to detect blood antigens in various fluids and tissues. Standard microscope slides were covered with 2 ml of

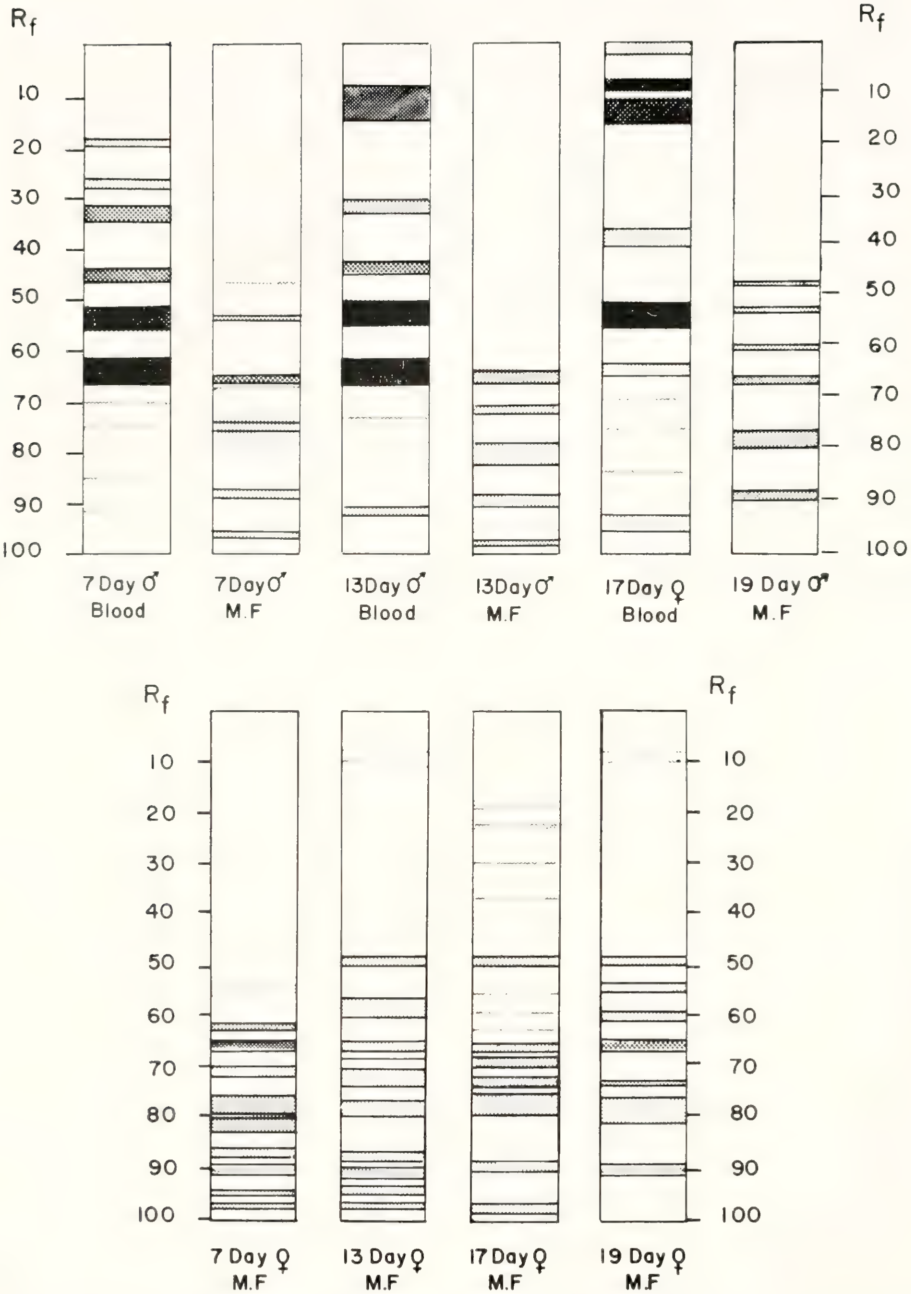
Rf
10
20
30
40
50
60
70
80
90
100
7 Day ♂ Blood
7 Day ♂ M.F
13 Day ♂ Blood
13 Day ♂ M.F
17 Day ♀ Blood
19 Day ♂ M.F
7 Day ♀ M.F
13 Day ♀ M.F
17 Day ♀ M.F
19 Day ♀ M.F

FIGURE 1. Typical acrylamide gel disc electrophorograms of blood and molting fluid proteins of Cecropia moths at various stages of adult development. In all cases 2 μl of blood or 10 μl of molting fluid were applied. Each drawing represents an actual gel. All samples were mixed with an equal volume of upper gel solution saturated with sucrose before photopolymerization.

1% agar (Difco, Special "Noble") in barbital acetate buffer at pH 8.6, $\mu = 0.025$. Troughs and holes were cut in agar as follows: 3 holes (1 mm diameter, 5 mm apart) and two troughs (6 mm apart) (Figs. 3 and 5). Electrophoresis was performed at 200 volts, applying 7 to 8 MA per slide for 60 minutes at 3° C. After electrophoresis, the separated proteins were reacted with rabbit sera against the blood of Cecropia pupae and adults. Precipitation arcs appeared after 12 to 48 hours at 25°, slides were washed in saline for three days, dried and stained with Buffalo Black.

Ouchterlony's double-diffusion analysis (Ouchterlony, 1958) was carried out in 5 cm Petri dishes covered with 4 ml of 1% agar in barbital acetate buffer. One central and 6 circumferential wells were cut out with a 13 gauge syringe needle and spaced 5 mm apart from each other (Fig. 6). Diffusion took place for 12 to 48 hours at 25° C.

Injection of dyes and proteins into the exuvial space and body cavity

Rabbit serum and purified rabbit gamma globulins were used as foreign proteins to help us detect macromolecular "traffic" between the exuvial space and the body cavity. Their presence in fluid compartments and in tissues was tested for by double diffusion analysis using sheep antiserum against rabbit gamma globulin. The distribution and penetration of macromolecules from one fluid compartment into another were also studied, using both rabbit serum proteins and molting fluid proteins stained with Buffalo Black. A series of dialysis studies with rabbit serum proteins stained with Buffalo Black demonstrated that, at concentrations of 0.1%, the dye remained firmly bound to the protein for at least 24 hours. This staining technique provided us with an easy way of tagging various proteins and studying their movements between fluid compartments. It was always confirmed by immunological methods.

Injection of foreign proteins, dye-"tagged" proteins or dye alone was made either into the exuvial space or into the body cavity in the dorsal side of the second or third abdominal segment.

Injections into the exuvial space posed no problem. However, special techniques were required to prevent blood and molting fluid from mixing during injections into the body cavity. In pharate adults younger than 14 days, the injection was made in the second or third abdominal segment through the pupal cuticle and directly into the heart. The epidermis in this region is closely pressed against the cuticle and no detectable mixing of blood and molting fluid occurred. In older pharate adults, the following procedure was carried out to prevent the mixing of blood and molting fluid during injection or immediately thereafter. A 6 mm square of pupal cuticle was excised between the second and third abdominal segment above the heart and the exposed edges of the pupal cuticle were sealed to the adult cuticle with melted paraffin. The small depression formed by this procedure was washed with saline and a volume of blood comparable to the amount of solution to be injected was removed from the heart. The experimental solution was introduced into the heart, and the exposed area sealed with paraffin.

RESULTS

Compartmentalization of the exuvial space

The first experiments were designed to determine whether compartmentalization exists within the exuvial space. About half of the molting fluid of the left antennal case of a 16-day old pharate adult was removed through an opening in the pupal cuticle and replaced by a mixture of molting fluid tagged with Buffalo Black. One day later, examination revealed that the stained protein had not diffused from the antennal case. In a second experiment, a similar injection was made into the left antennal case of a 20-day old pharate adult. Five hours after the injection, the Buffalo Black-tagged protein had spread from the left antennal case to the fore and hind wings, head and thorax.

In another experiment on a 20-day old pharate adult 1 ml of 1% aqueous solution of Buffalo Black was injected into the exuvial space at the tip of the abdomen. Ten hours after the injection, the Buffalo Black-tagged protein had diffused into the wings, thorax and head.

These experiments were repeated several times with the same results. Clearly, the exuvial space is compartmentalized and the fluids in the compartments do not mix with one another until several days before emergence. For this reason, all samples of molting fluid used in this study were withdrawn from the same place, namely the tip of the abdomen. Apparently, molting fluid is formed separately in several regions between which parts of the pupal cuticle remain attached to the epidermis. These areas of persistent attachment effectively divide the exuvial space into compartments. Just prior to ecdysis, the pupal cuticle detaches in these areas, and the exuvial space becomes continuous.

The protein composition of blood and molting fluid as revealed by disc electrophoresis

Samples of blood and of molting fluid were taken from pharate adults at various stages of the pupal-adult transformation and from diapausing pupae. The samples were subjected to disc electrophoresis and some typical results are recorded in Figure 1. From an analysis of the gels illustrated in this Figure and more than 50 similar gels, the following facts emerged:

(1) The molting fluid was not contaminated by blood, because it was colorless. The slightest contamination could be detected by the occurrence of yellow pigments. Also, as Figure 1 shows, several of the densest blood protein bands (R_f 34 and 45) were absent from molting fluid.

(2) None of the major protein bands in the blood was a major component of the molting fluid and vice versa. Band R_f 66, a major blood protein component, was detectable in low concentrations in the molting fluid. All 5 major blood protein bands had R_f's between 0 and 66 while major molting fluid protein bands had R_f's greater than 66.

(3) Molting fluid contained at least 10 proteins which were not detected in the blood.

(4) Profound changes occurred in the protein pattern of the blood and molting fluid during development. Only changes that occurred in the molting fluid will

be discussed here. The molting fluid on day 7 is of special interest because it is still inactive enzymatically and does not contain products of cuticular digestion. It contained 8 to 10 distinct protein bands, but none had R_f's less than 45. However, between day 13 and 17, a number of new bands appeared with R_f's ranging from 10 to 38, some of which persisted throughout development. Whether these proteins have anything to do with the enzymatic activity of the molting fluid, or include products of cuticular digestion, will be considered in the discussion.

(5) A number of bands in blood and molting fluid have similar R_fs. Which of these bands represented identical proteins is considered in the next section.

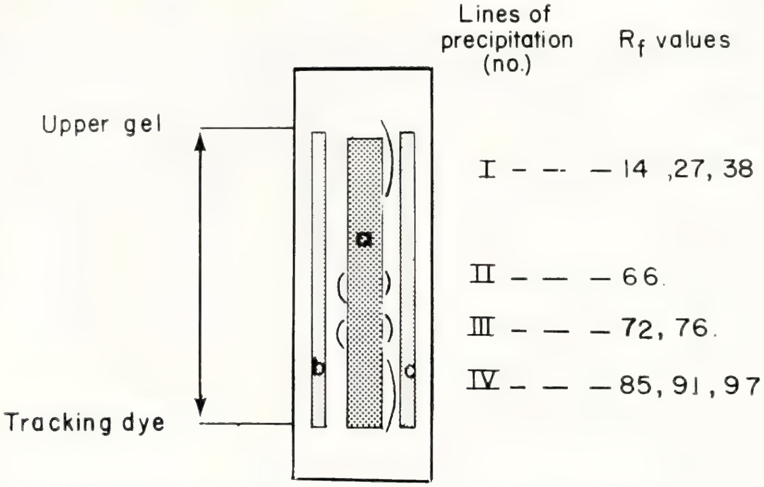

FIGURE 2. Diagram showing results of combined disc electrophoresis and immunodiffusion of molting fluid of 17-day old developing adult male Cecropia moth. The results reveal four lines of precipitation (I–IV) indicating the presence of four blood antigens in the molting fluid. The R_f values of the molting fluid protein bands which correspond to the lines of precipitation are indicated. It was not possible to determine which of several protein bands corresponded to each line of precipitation. Stipples indicate (a) unfixed half of acrylamide gel; (b) rabbit antiserum against blood of male pupae; and (c) rabbit antiserum against blood of male adults.

Proteins common to molting fluid and blood

The following electrophoretic and immunological methods were used to determine which protein bands with similar R_fs in molting fluid and blood are identical.

Separation by disc electrophoresis and precipitation by antisera against blood proteins: Thirty μl of molting fluid from 17-day old pharate adult males were applied to each acrylamide gel and the proteins separated as previously described. Following electrophoresis, the gels were cut longitudinally into two halves; one was stained with Buffalo Black to locate the protein bands and the other half was placed on a microscope slide coated with 2% agar. Rabbit antisera against male pupal blood and against male adult blood were added into two parallel troughs on each side of the split gel. Diffusion was allowed to occur until no new lines of precipitation appeared. The results are recorded in Figure 2. Four lines of precipitation were formed with antiserum against adult blood serum and two with antiserum against pupal blood. The R_f values of the molting fluid protein bands which could correspond to each of these lines are recorded in Figure 2. It is impossible to decide precisely which of these bands are the blood antigens.

From this result it is clear that the molting fluid of 17-day old pharate adults contains 4 antigens found in adult blood. Two of these antigens are absent from pupal blood or present in much lower concentrations. Further support for these conclusions comes from immunoelectrophoretic data.

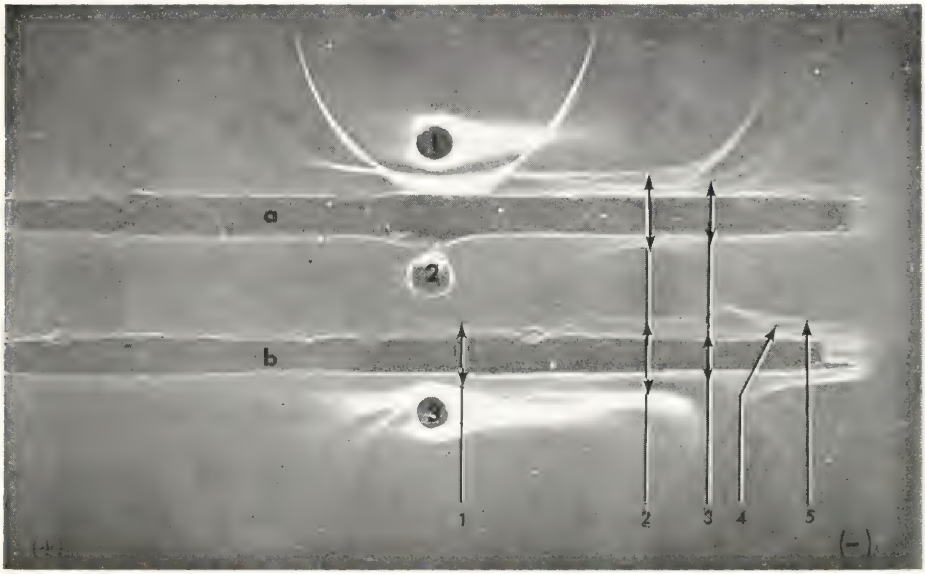

FIGURE 3. Immunoelectrophoretic analysis of blood and molting fluid of 16-day old developing adult male Cecropia moth. The wells contained (1) 2 μl of blood, (2) 10 μl of molting fluid, and (3) 2 μl of blood. The longitudinal troughs had (a) rabbit antiserum against blood of male pupae, and (b) rabbit antiserum against blood of male adults. The numbered arrows indicate the lines of precipitation formed by blood antigens present in the Cecropia blood and molting fluid.

Immunoelectrophoresis: Molting fluid and blood from pharate adults 13, 16, 19 and 20 days old were analyzed by precipitation with rabbit antisera against the blood of male pupae and against the blood of female pupae. Figure 3 shows a typical result obtained with a 16-day old pharate adult. The many lines formed by the blood need not concern us. The key result is the demonstration that molting fluid formed two lines of precipitation with antiserum against pupal blood and five lines with antiserum against adult blood. These lines corresponded to those formed by blood antigens as follows:

(1) Lines 2 and 3 formed with antiserum against male pupal blood. A diffuse line, No. 1, also formed but is not visible in the photograph.

(2) Lines 1 to 5 formed with antiserum against male adult blood. There was

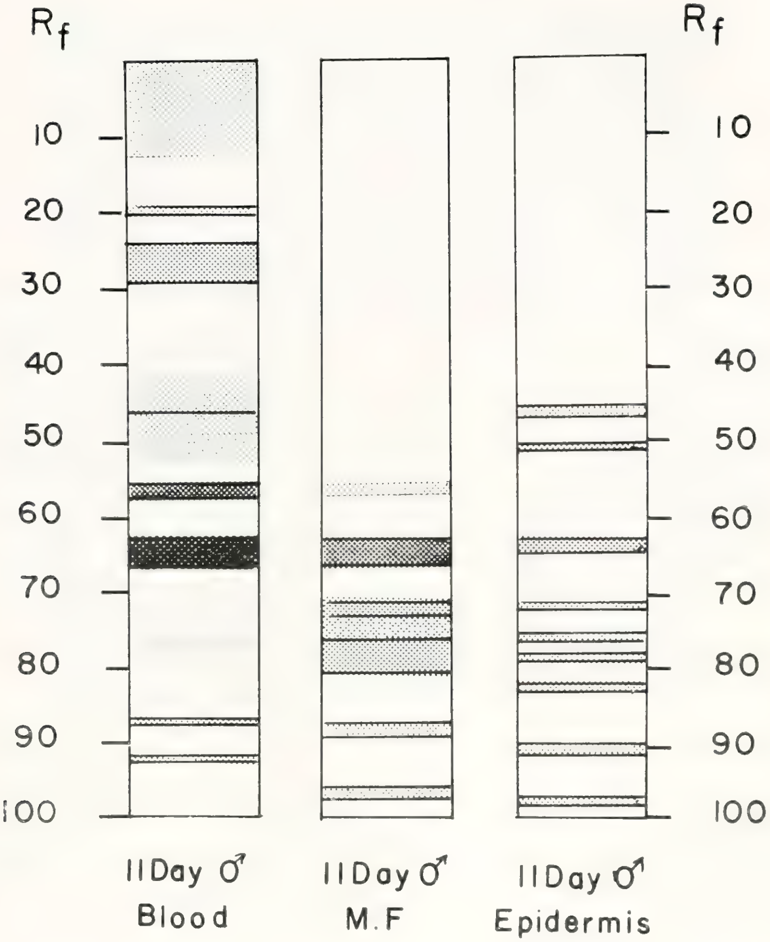

FIGURE 4. Acrylamide gel disc electropherograms of blood, molting fluid and epidermis of an 11-day old, pharate, adult male Cecropia moth.

no basic change in the number or identity of the blood antigens present in molting fluid during the final seven days of pharate adult development. These results confirm and extend the combined disc electrophoresis-double diffusion experiments described above.

Ouchterlony double diffusion analysis confirmed the identity between several blood antigens and molting fluid antigens (*cf.* also results presented in section 4(c) below and Figure 6).

The origin of proteins common to blood and molting fluid

It is generally believed that the epidermis is "the source of at least the principle constituents of the molting fluid" (Passonneau and Williams, 1953). To deter-

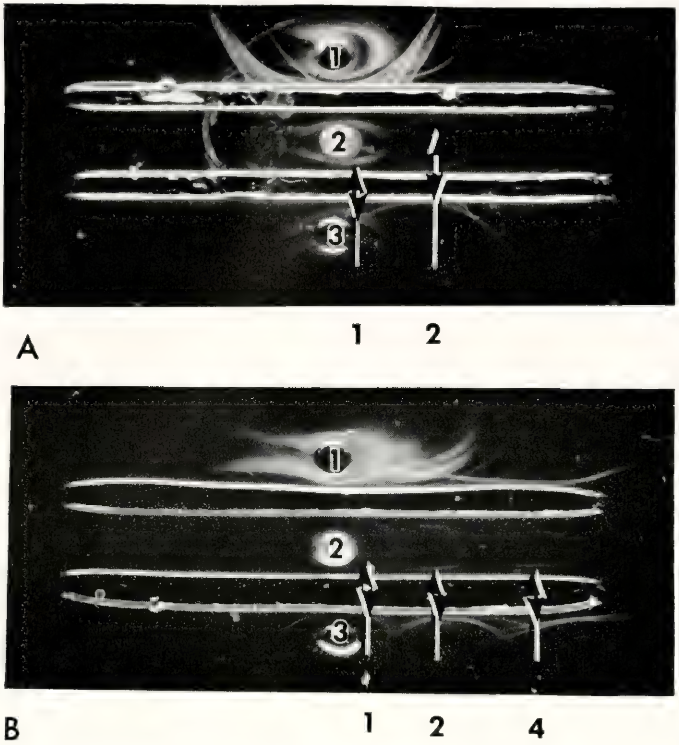

FIGURE 5. Immunoelectrophoretic analysis of blood, molting fluid and epidermis of 11-day old, pharate, adult male Cecropia moth. The wells contained (1) 2 μl of blood, (2) 10 μl of epidermal homogenate, and (3) 10 μl of molting fluid. In (A) rabbit antiserum against blood of male pupae was in both troughs, and in (B) rabbit antiserum against blood of male adults in both troughs. The numbered arrows indicate the lines of precipitation formed by Cecropia blood antigens. Lines 3 and 5 did not show up in the photograph.

mine whether the epidermis was the source of the five antigens found in both the molting fluid and blood, epidermal proteins were examined by disc electrophoresis, immunoelectrophoresis and double diffusion.

Figure 4 records the results of disc electrophoresis of epidermis, blood and molting fluid from 11-day old pharate adults and demonstrates that the epidermis and molting fluid have a number of proteins with the same R_f values. They both lack the same major blood proteins. In short, in terms of disc electrophoresis patterns, epidermis resembles molting fluid more than blood.

The identity between certain epidermal, molting fluid and blood proteins was established by the following immunoelectrophoretic analyses. Blood, molting fluid and epidermis from 11-day old pharate adults were subjected to immunoelectrophoresis using antisera against male pupal blood and male adult blood. It is evident from the results in Figure 5 that blood antigens 1, 2 and 3 were present both in the epidermis and the molting fluid when reacted with antiserum against pupal blood. Lines of precipitation 1, 2, 4 and 5 were formed by the epidermis and lines 1 to 5 by the molting fluid following their reaction with antiserum against adult blood. The yellow pigments characteristic of both pupal and adult blood, which are bound to certain blood proteins, were absent from the abdominal epidermis as well as from the molting fluid of pharate adults.

These observations show that epidermis contains at least 4 of the 5 blood antigens found in the molting fluid. Proof of the identity among the antigens in the epidermis, blood and molting fluid was established by Ouchterlony double-diffusion analysis as follows: Molting fluid, blood and abdominal epidermis of 11-day old pharate male adults were examined by the Ouchterlony double-diffusion technique for the presence of lines of identity between these three protein mixtures. Rabbit antisera against male pupal blood and male adult blood were used. The results in Figure 6 reveal that two identical lines of precipitation were formed by the molting fluid, epidermis and blood when tested with antiserum against pupal blood. Figure 6 also shows that at least two identical lines were formed by blood, molting fluid and epidermis when reacted with antiserum against adult blood. In both cases heavy lines of precipitation were formed only by the blood, and were absent from the epidermis and molting fluid.

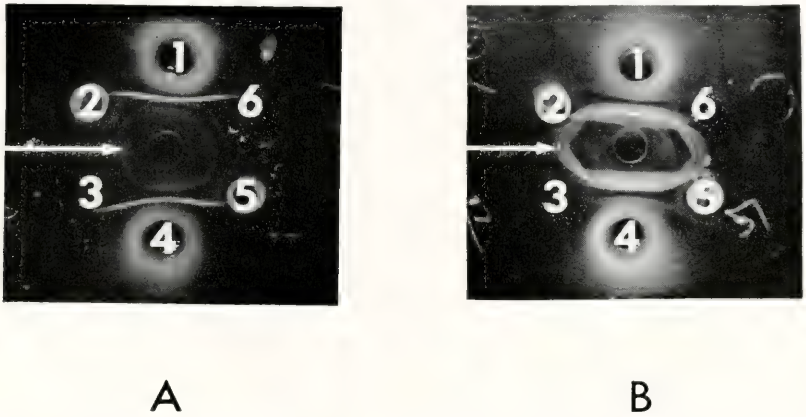

FIGURE 6. Double diffusion analysis of blood, epidermis and molting fluid of 11-day old, pharate, adult moth absorbed with two different antisera. Surrounding wells were prepared with (1, 4) blood, (2, 5) abdominal epidermis, and (3, 6) molting fluid. In (A) the center well contains rabbit antiserum against male pupal blood, and in (B) the center well contains rabbit antiserum against male adult blood. Blood antigens present in these three protein mixtures which fuse and form lines of identity are indicated by arrows. Another line of identity in (A) between wells 2 and 3 and between wells 5 and 6 did not show up in the photograph.

Although the resolution of the double-diffusion technique is much less than that of immunoelectrophoresis, these observations are consistent with the results of immunoelectrophoresis and establish that several of the blood antigens present in molting fluid are identical with those present in the epidermis.

The route of entry of blood antigens into molting fluid

The occurrence of blood antigens in molting fluid raises the question of how they get there. Are they secreted by the epidermal cells into the exuvial space and also into the blood? Or, do they originate in some other tissue, to be transported by epidermal cells into the exuvial space? Indeed, this raises the more general question of what sort of commerce there is between the molting fluid and blood compartments of the insect.

To answer this question, rabbit whole serum and rabbit whole serum tagged with Buffalo Black was injected into the abdominal exuvial space or into the body cavity of pharate adult females of different ages. Double diffusion analysis with sheep antiserum against rabbit gamma globulin was used to detect the presence of the gamma globulin fraction of the injected serum in various fluid compartments and tissues. The distribution of Buffalo Black-tagged proteins was visually determined. Unless otherwise indicated each experiment was repeated on three animals.

Injection of rabbit serum proteins into the exuvial space: In the first experiment 200 μl of molting fluid was removed from 7-day old pharate adults. Following this, 150 μl of rabbit serum was injected into the exuvial space and the opening sealed with paraffin. Four or ten days after the injection, the animals were sacrificed and blood and molting fluid were analyzed by double diffusion. The results revealed that rabbit gamma globulins were detected in the molting fluid but not in the blood. Apparently, in 11- to 17-day old pharate adults, the rabbit serum proteins did not move from the exuvial space into the blood. Also, the rabbit serum proteins retained their antigenic activity for at least ten days in the molting fluid, notwithstanding the presence of proteolytic enzymes in this fluid (Passonneau and Williams, 1953).

In another experiment, between 20 and 100 μl of whole rabbit serum protein were injected into the exuvial space of 19-day old pharate adults at the peak of molting fluid absorption. Twenty-four hours later, blood and molting fluid were tested by double diffusion analysis for the presence of rabbit serum proteins using sheep antiserum against whole rabbit proteins and sheep antiserum against rabbit gamma globulin. No rabbit serum proteins were detected in the blood, but they were found in the molting fluid.

In a third experiment 250 μl of whole rabbit serum was injected into the exuvial space of a 20-day old pharate adult. The insect resorbed its molting fluid four days later, whereupon blood, fat body and ovaries were examined by double diffusion analysis. No lines of precipitation were observed. Apparently, immunologically active rabbit serum proteins are not resorbed into the blood even when active molting fluid absorption takes place.

To summarize, foreign proteins injected into the exuvial space of pharate adults of different ages could not be detected in the blood of these animals by immunological methods. This finding indicates that macromolecules similar to rabbit gamma globulins do not penetrate the new adult cuticle and epidermis to enter the blood, prior to or during active molting fluid absorption.

Injection of rabbit serum proteins into the body cavity: The next question asked was whether macromolecules could cross the epidermis from the body cavity into the exuvial space. To answer this question, rabbit serum proteins were injected into the heart of pharate adults of different ages.

In the first experiment, 7-day old pharate adults were injected with 100 μl of rabbit serum and killed 4, 10 or 15 days thereafter. Double diffusion analysis of molting fluid, fat body and blood revealed rabbit gamma globulins only in the blood. Thus, although rabbit globulins were detected in the blood for 15 days after injection, these proteins failed to pass from the blood into the exuvial space at any stage.

A similar experiment was performed on 14-day old pharate adults in which 350 μl of rabbit serum protein tagged with Buffalo Black were injected. The pharate adults were killed five days after the injection, and blood, molting fluid and various tissue were examined visually for the presence of blue dye, and immunologically by double diffusion, for the presence of rabbit serum proteins. The results revealed that the oocytes were the only tissue, besides blood, which contained gamma globulins. The oldest oocytes did not stain blue, but younger ones did, and the oocytes as a whole contained gamma globulins. However, when gamma globulins were injected on days 19 or 20, none of the oocytes took up gamma globulins. Although the Malpighian tubules were stained blue, no antigenically active gamma globulins were detected. Apparently the blue color is due to dye molecules which dissociated from rabbit serum proteins that might have been degraded.

Taken together, these experiments indicate that foreign proteins do not penetrate from the exuvial space to the blood or vice versa. The presence of certain native proteins in the blood and their absence from the molting fluid, coupled with the presence of certain native proteins in the molting fluid and their absence from the blood, also demonstrates an effective separation between the two fluid compartments. The results suggest that the blood antigens found in the molting fluid are not transported from the blood into the molting fluid, but are secreted by the epidermis directly into both molting fluid and blood.

Sites of molting fluid absorption

To identify the sites of molting fluid absorption, the fate of Buffalo Black-tagged molting fluid was investigated. In pharate adults, molting fluid is absorbed on days 19 to 21. A series of developing adults 17 to 18 days old had the pupal cuticle on the tip of the abdomen removed, 0.1 ml of molting fluid was collected and mixed with 0.9 ml of 2% Buffalo Black in saline. The resulting mixture was reinjected into the exuvial space. Four days later, after emergence, the adult integument was examined. Only one part of the abdominal integument showed conspicuous blue staining. This was a narrow band located on each intersegmental membrane close to the sclerotized ridge at the posterior margin of each segment (Fig. 7). This blue-staining band was made up of many small pits or depressions about 15 microns in diameter. These pits were the only part of the cuticular surface that stained blue (Fig. 8) and from which diffusion of blue dye down and laterally into the integument took place.

In another experiment pupal cuticle was removed from 19- and 20-day old pharate adults and a drop of Buffalo Black-tagged molting fluid was applied to various parts of the adult integument. The penetration of the blue dye through the pits could be followed under the microscope. Within 15 minutes the dye was absorbed through the pits and its centrifugal diffusion into the integument could be followed. It was absorbed only through the pits.

These pits absorbed Buffalo Black-tagged molting fluid only prior to ecdysis. If Buffalo Black-tagged molting fluid was applied to the integument after ecdysis, the dye-protein mixture accumulated *on* the pits, but could be washed away with saline. It failed to enter the integument, presumably, because of a waterproof coating on the pits.

Similar pits are also found in corresponding parts of larvae and pupae. They are permeable to Buffalo Black-tagged molting fluid only before ecdysis. In fifth-stage larvae of *Samia cynthia ricini,* for example, five to six hours after ecdysis, the pits failed to absorb Buffalo Black-tagged molting fluid. In prepupae of *Galleria mellonella* Buffalo Black-tagged molting fluid was also absorbed via pits, but after pupation there was no absorption.

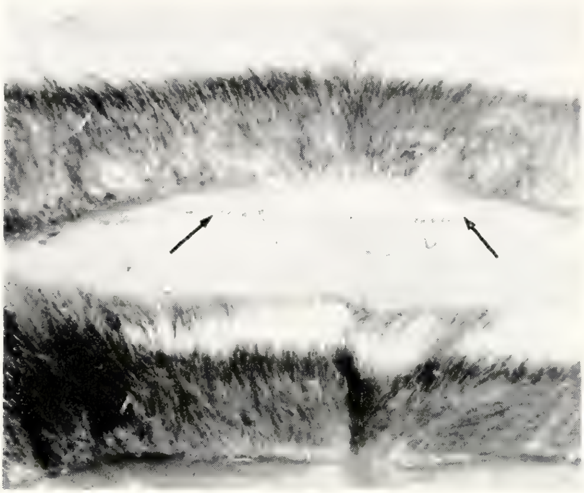

FIGURE 7. Portion of dorsum of abdomen of newly emerged female Cecropia moth showing tonofibrillar insertions in the intersegmental membrane (arrows). Buffalo Black dye was applied to the surface of the cuticle to emphasize the bands of pits for photographic purposes (× 16).

These pits appear to be principal sites of molting fluid absorption in the abdomen. In the thorax, molting fluid is resorbed on the patagia, parapatagia, membranes at the bases of the legs and also in the membranes lying between epimeron 2 and 3 and subalare 2 and 3. In the head, some Buffalo Black-tagged molting fluid appeared to be sucked into the mouth and some was also absorbed by the integument beneath the mouth parts.

These pits mark the points at which tonofibrils occur. In the abdomen, the longitudinal muscles attaching adjacent segments and the lateral muscles within a segment make tonofibrillar insertions in these pits. In pharate adult Cecropia, the small portion of each tonofibril which attaches the pupal cuticle to the adult cuticle (and due to which the pharate adult is capable of moving the pupal cuticle) breaks down after about day 18. After this time, the absorption of molting fluid takes place through these pits in the adult integument.

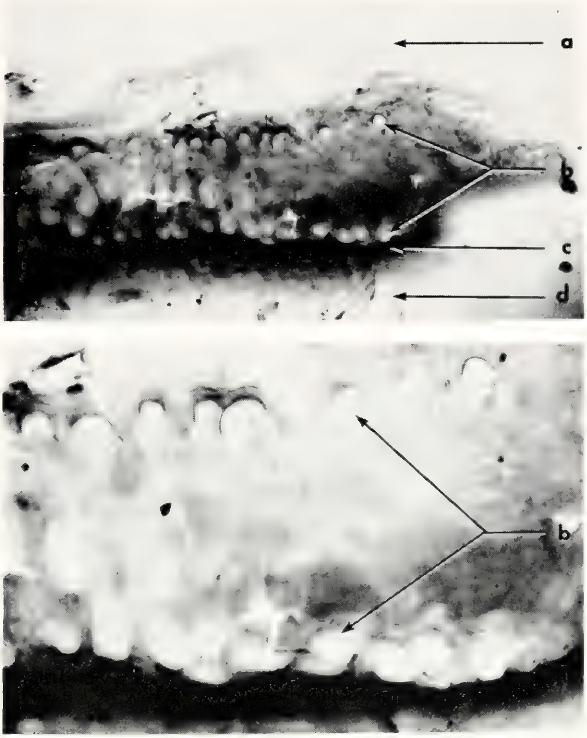

FIGURE 8. Tonofibrillar insertions showing absorption of stained molting fluid proteins in a portion of the intersegmental membrane of a 21-day old, pharate, male Cecropia moth. Approximately 250 μl of molting fluid tagged with Buffalo Black had been injected on day 18 into the exuvial space. Three days later, the animal was killed and a portion of the abdominal dorsum was free of adhering tissues and fixed. An overall view of a portion of a segment (× 320) is shown above (A), and a high power view of above (× 800) below (B). Labels indicate: (a) intersegmental membrane, (b) tonofibrillar insertions or "pits," (c) the sclerotized ridge, and (d) a segment showing scale sockets.

These experiments with Buffalo Black-tagged protein suggest that some macromolecules are absorbed from the molting fluid. Proof that this occurs was provided by the following immunological experiment. An injection of 275 μl of rabbit serum was made into the ecdysial space of an 18-day old developing adult. When it had emerged five days later the integument (adult cuticle plus epidermis) was freed of surrounding tissues and scales and washed thoroughly in saline. It was then homogenized and tested for the presence of rabbit serum proteins by double diffusion analysis. The results revealed rabbit serum proteins in the integument, while longitudinal muscles which are attached to the tonofibrils showed no reaction. Identical results were obtained with rabbit serum proteins tagged with Buffalo Black. These observations prove that the integument absorbs macromolecules along with molting fluid and these remain there for several days in an immunologically active state. The results indicate that in the normal process of absorption of molting fluid some macromolecules may be absorbed intact.

Principal sites of absorption in the abdomen are the tonofibrillar insertions. The absorbed proteins do not appear to enter the blood but some of them enter the integument.

DISCUSSION

The results indicate that molting fluid is a separate fluid compartment and has at least 10 protein components which were not detected in the blood. These 10 components are not products of cuticular digestion since they are present in inactive molting fluid prior to cuticular digestion. The blood and molting fluid do share five antigens. One of these, a major blood protein band, is a minor component of molting fluid. The other four common protein components are minor compartments of both blood and molting fluid.

This different protein composition in the two fluid compartments depends on the epidermal cells which act as a barrier to most macromolecules in the molting fluid and the blood. The existence of this barrier is evident from the fact that numerous native blood proteins were never detected in the molting fluid, and many native molting fluid proteins were never detected in the blood. Moreover, Buffalo Black-tagged molting fluid proteins also failed to appear in the blood. Similar results were obtained with various rabbit serum proteins of different molecular weights which failed to cross from one fluid compartment to another at any stage of adult development.

The fact that the epidermis acts as a barrier to many macromolecules suggests that most molting fluid proteins are synthesized by the epidermis itself and are not synthesized elsewhere and transported to the epidermis. This being the case, the presence of five common antigens in both molting fluid and blood suggests that, either (a) the epidermis secretes certain proteins in only one direction, but can secrete other proteins (the five blood antigens) in both directions; or (b) the epidermis secretes the five antigens into the molting fluid and other tissues secrete the same proteins into the blood; (c) the common antigens are secreted by some other tissues into the blood and are transported by the epidermis into the molting fluid. We favor the first suggestion.

These observations lead us to question earlier conclusions that molting fluid contains blood (*e.g.*, Jeuniaux, 1958). It seems likely that, in those cases where molting fluid contains blood, some damage to the delicate new cuticle and epidermis occurred which caused mixing. Indeed, the only *bona fide* cases where blood is to be expected as a normal component of the molting fluid are in insects like *Sialis* in which the larval gills with enclosed blood are trapped in the exuvial space at the time of pupation (Selman, 1960).

It is also worth noting, that, unless great precautions are taken, it is difficult to obtain blood from pharate adult insects without contaminating it with molting fluid. There have been several reports of proteins that appear in the blood only at the time of molting (*e.g.*, McCormick and Scott, 1966). One wonders whether some of these molt-connected blood proteins might be molting fluid proteins that contaminated the blood in the course of collecting blood samples.

As far as the exchange of small molecules between blood and molting fluid is concerned, we have no new data. Wigglesworth (1933) and Jeuniaux (1958) indicate that dyes of molecular weights up to at least 500 can move from the molting fluid to the blood and Passonneau and Williams (1953) have shown that glycine $-C^{14}$ readily moves from the exuvial space into tissues. Locke (1966) has presented evidence that small molecules are absorbed through small 30 Å pores in the cuticulin of *Calpodes*.

The change in protein composition of molting fluid during adult development requires comment. Recent electron micrographic studies of molting in Apterygota indicate that molting fluid initially consists of granules with inactive enzymes which later become active (Noble-Nesbitt, 1963a, 1963b). The mechanism of this activation is not understood. In the present experiments, there was a great increase in the number of protein bands in molting fluid during the last eight days of adult development, at which time the molting fluid becomes activated. Whether these new bands represent newly-secreted proteins or enzymes, a rearrangement (activation?) of previously secreted proteins, or some cuticular breakdown products is not yet known.

The mechanism for absorption of the molting fluid during the final two days of adult development is unknown. Contrary to the generally held view, the absorption does not take place through the integument as a whole, but through particular regions. In the head and thorax, these regions are principally various flexible membranes at the bases of the appendages. In the abdomen, the principal sites of resorption are pits which represent the points through which tonofibrils make attachment to the old cuticle. In all cases in which it has been studied carefully, the tonofibrils remain attached to the old cuticle until just before ecdysis (Lai-Fook, 1966; Noble-Nesbitt, 1963a, 1963b; Wachter, 1930; Wolfe, 1954). This appears to be true also in developing adult Cecropia. Until the tonofibrils break, the old and new cuticles are closely bound together at the points of attachment. On about day 19, the attachments between the tonofibrils and the pupal cuticle ruptures, and the adult can now move *within* the old pupal cuticle. The cause of this rupturing is not clear, however. As a result of the rupturing, the part of the tonofibrils connecting the two cuticles detaches from the new adult cuticle and leaves the point of attachment on the new cuticle exposed. It is through this exposed surface of adult cuticle—the pit—that molting fluid is absorbed.

The particular ultrastructural features of the tonofibrils that make them suitable sites for resorption requires further study (*cf. e.g.,* Auber, 1963; Bouligand, 1962). Perhaps it is simply that by day 19 most of the cuticle is covered with substances that render it impermeable to molting fluid, whereas the newly exposed cuticular surfaces of the pits are permeable to macromolecules. In any event, although the epidermis on the general surface of the abdomen may be able to absorb molting fluid, in practice, the principal sites of absorption are the pits.

It has been demonstrated previously that, after molting fluid absorption, some molting fluid proteins remain on the surface of the adult cuticle after ecdysis (Wolfe, 1954; Jeuniaux, 1957). However, so far as we are aware no one has demonstrated that macromolecules in the molting fluid get absorbed. The present experiments with rabbit serum proteins demonstrate absorption of foreign proteins from the molting fluid and also demonstrate that these proteins end up *in* rather than *on* the integument. Whether they are in endocuticle, epidermis or both was not determined.

We wish to thank Dr. Narayan Patel and Mrs. Ilona Polony for performing a number of disc electrophoretic separations and Mr. Gordon Marsh for his helpful comments on the typescript. This research was supported in part by research grants, HE 06320 and HE 10082 from the United States Public Health Service.

SUMMARY

1. Molting in insects is always accompanied by the production of a molting fluid which fills the exuvial space between the new and the old cuticle and digests the inner layers of the old cuticle. In *Hyalophora cecropia,* molting fluid is secreted at the outset of adult development and persists until two days before eclosion, whereupon it is absorbed.

2. The present report examines the protein composition of the molting fluid of Cecropia, the origin of the molting fluid proteins, the relation of these proteins to blood proteins and the exchange of macromolecules between the molting fluid and the blood. It also examines the sites of absorption of molting fluid.

3. Disc electrophoresis on acrylamide gels reveals that the molting fluid of Cecropia contains about fifteen protein bands which can be resolved at pH 8.6. Some of these protein bands are detected in the molting fluid at all stages, whereas others appear only at specific times. About ten of the bands are peculiar to molting fluid and are not detected in the blood. About five bands are detectable in both blood and molting fluid, but none of these common bands appears to be a major component of the molting fluid, and only one is a major blood protein. In contrast, the epidermis contains most of the major protein bands found in molting fluid but lacks all but one of the major protein bands present in the blood.

4. Immunological analysis reveals that blood and molting fluid share five antigens. At least four of these common antigens also occur in the epidermis which appears to secrete these antigens into both the molting fluid and the blood.

5. Native and foreign proteins do not penetrate from the exuvial space into the blood or vice versa. Apparently the epidermis and cuticle act as a barrier to the exchange of most macromolecules between the blood and molting fluid. The exuvial space is clearly a separate fluid compartment.

6. In addition the exuvial space itself is compartmentalized and the fluids in the compartments do not admix several days before eclosion.

7. Absorption of molting fluid during the final two days of adult development occurs most readily through particular regions of the integument. In the abdomen the principal sites of absorption are pits which represent the points through which tonofibrils make attachment to the old cuticle. Two days before ecdysis, the attachments between the tonofibrils and the pupal cuticle rupture, exposing the points of attachment on the new cuticle. It is through these exposed surfaces that much of the molting fluid is absorbed. Molting fluid is also absorbed in the head and thorax through various flexible membranes at the bases of the appendages.

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REVERSIBLE RESPONSE TO PUROMYCIN AND SOME CHARACTERISTICS OF THE UPTAKE AND USE OF AMINO ACIDS BY UNFERTILIZED SEA URCHIN EGGS[1]

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Several groups have reported that all unfertilized sea urchin eggs incorporate labeled amino acids into protein, as judged by autoradiography (Bell and MacKintosh, 1967; MacKintosh and Bell, 1967; Epel, 1967; Tyler, Tyler and Piatigorsky, 1968). However, when incorporation is measured biochemically (*i.e.* by homogenization and acid precipitation of a radioactively labeled sample containing 1000 to 10,000 eggs) there is a strong theoretical possibility that a few "immature" eggs or ovarian fragments in the preparation could be contributing to it heavily. Thus experiments relying on measurement of the rate of incorporation in these preparations as a measure of the rate of protein synthesis in mature unfertilized eggs (MacKintosh and Bell, 1967; Epel, 1967) could be in substantial error, as suggested by Stavy and Gross (1967).

That it is not in error emerges from some basic facts presented in this work about the uptake and incorporation into protein of amino acids by unfertilized eggs. The basal level of incorporation in unfertilized egg preparations is, in fact, the rate of incorporation by mature unfertilized eggs, and is not attributable to contamination with immature cells. Support for this conclusion is based heavily on the differential response of unfertilized eggs as compared with oocytes to puromycin; the effect of the antibiotic on the former is reversible, while on the latter it is not. It is also based on other data which is presented below.

MATERIALS AND METHODS

Animals and gametes

Specimens of *Strongylocentrotus purpuratus* were obtained from Pacific Bio-Marine, Venice, California. Specimens of *Arbacia punctulata* were obtained from Mr. Norris Hill, Beaufort, North Carolina. Animals were maintained at about 12° C in aerated tanks with subsand filters, and were used or discarded within two weeks after arrival.

Gametes were obtained by injection of isotonic (0.53 M) KCl, a method which yielded egg preparations that were superior (in terms of freedom from contaminating ovarian material) to those obtained by excision of gonads.

Eggs were prepared for use by passing them through four layers of cheesecloth, washing twice by settling in Millipore filtered sea water, and suspending them in

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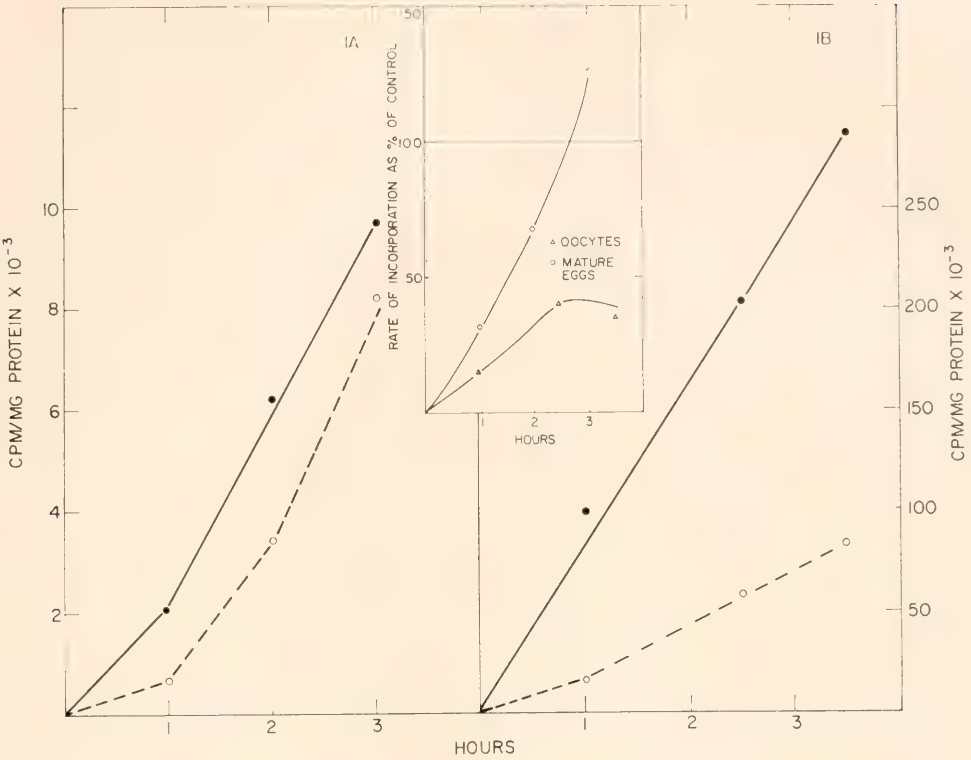

FIGURE 1. (a) Mature eggs of *Arbacia* were collected as described in Materials and Methods. Half were treated with puromycin at 235 μg/ml for four hours, while controls remained in untreated sea water. Both groups were rinsed and placed in fresh sea water, and maintained for an additional hour. ^{14}C amino acids were then added to both groups (1 μc/ml) and samples were withdrawn from both at hourly intervals for analyses as usual. Data are presented as acid insoluble cpm/mg protein for controls (●——●) and puromycin treated (○– – –○). Samples were counted at 11% efficiency. (b) An egg suspension containing 30% oocytes (eggs with a germinal vessel) was prepared by mincing the gonads of a female of *Arbacia* after it had been induced by KCl injection to shed most of its mature eggs. The preparation was divided in half and treated exactly as above, except that puromycin treatment was for only 1.5 hours, and radioactivity was counted at 90% efficiency. The data of Figure 1a and 1b is summarized in the inset, recalculated as rate of incorporation relative to controls, and plotted together for comparison of mature eggs (○——○) and oocytes (△——△).

Millipore filtered sea water containing penicillin (160 μg/ml) and streptomycin (100 μg/ml). Eggs were maintained at 20° C (*Strongylocentrotus*) or at room temperature (*Arbacia*), which varied from day to day between 21° and 24° C, in Erlenmeyer flasks on a rotary shaker at 60 to 70 rpm.

To determine their maturity and condition, we examined egg preparations microscopically before use, and discarded any with an excessive number of oocytes, fragments of ovaries or fertilized eggs. Oocytes are easily detected by the presence of a large germinal vessicle. Typical levels of contamination with oocytes were of the order of 0.1 to 0.3 per cent. Bacterial contamination was occasionally monitored by plating samples of the egg suspension on agar made up in 80 per cent sea water and 20 per cent Charity Waymouth medium. Typical levels of contamination within five or six hours of the start of an experiment were of the order of 10–20 viable bacteria per ml. The maximum found was 1280 bacteria per ml, after over 24 hours of incubation of a culture. On the most gratuitous possible assumption a single bacterial cell could not yield more than 0.2 DPM (if uniformly labeled with ^{14}C at 50 mc/mMole). Thus in no case could contamination represent more than 3% of the radioactivity of a sample, and in most cases the amount would be far smaller than this. This level of contamination is not significant. Tyler, Tyler, and Piatigorsky (1968) also report negligible bacterial contamination.

TABLE I

Uptake of ^{14}C amino acids by eggs after short exposure and after rinsing

Exposure time	No rinse	1 rinse	4 rinse
3 min	—	3,900	—
3 min	—	4,050	—
1 hr	106,000	94,100	94,700
1 hr	102,000	97,000	95,600

Eggs of *Strongylocentrotus* were labeled for times indicated with 1 $\mu c/ml$ ^{14}C amino acids. "No rinse" eggs were pipetted into 10 volumes of sea water (20° C) in a conical centrifuge tube, sedimented, resuspended and transferred to a clean tube to be dissolved in 8 M urea withuot further rinses. "1 rinse" consisted of suspending "no rinse" eggs in 2 ml cold homogenization medium and resedimenting and dissolving in 8 M urea. "4 rinses" consisted of three sea water washes prior to the wash with homogenization medium. Data are cpm/mg protein in the homogenates. Each figure represents a single determination on a single homogenate. All samples came from the same egg preparation.

Assay of incorporation of radioactivity, even at the maximum observed levels of contamination

^{14}C labeled reconstituted protein hydrolysate (a mixture of L amino acids with an average specific activity of about 140 mc/mMole, obtained from Schwarz Bioresearch, Orangeburg, New York) was used as a protein precursor. To measure incorporation in eggs which had been exposed to labeled precursor, a 0.5 or 1.0 ml sample of egg suspension was withdrawn from the incubation vessel and pipetted into a 12 ml conical centrifuge tube containing 10 ml of ice cold sea water. The eggs were sedimented by a brief centrifugation (about 5 seconds at full power in a clinical centrifuge) and the supernatant withdrawn. The eggs were then rinsed once with 2 ml cold homogenization medium [0.25 M sucrose, 0.24 M NH_4Cl, 0.01 M $MgCl_2$, 0.01 M Tris, pH 7.4; described by Spirin and Nemer (1965)] and dissolved overnight in 8 M urea. The urea treatment was found to render soluble the radioactivity which was hot acid labile, *i.e.*, such non protein incorporation as amino acyl t-RNA labeling. The urea homogenate was routinely analyzed as follows: a portion was precipitated by the addition of bovine serum albumin as carrier if needed and 30 per cent (w/w) TCA to give a final concentration of 15 per cent TCA and the precipitate collected on Millipore filters

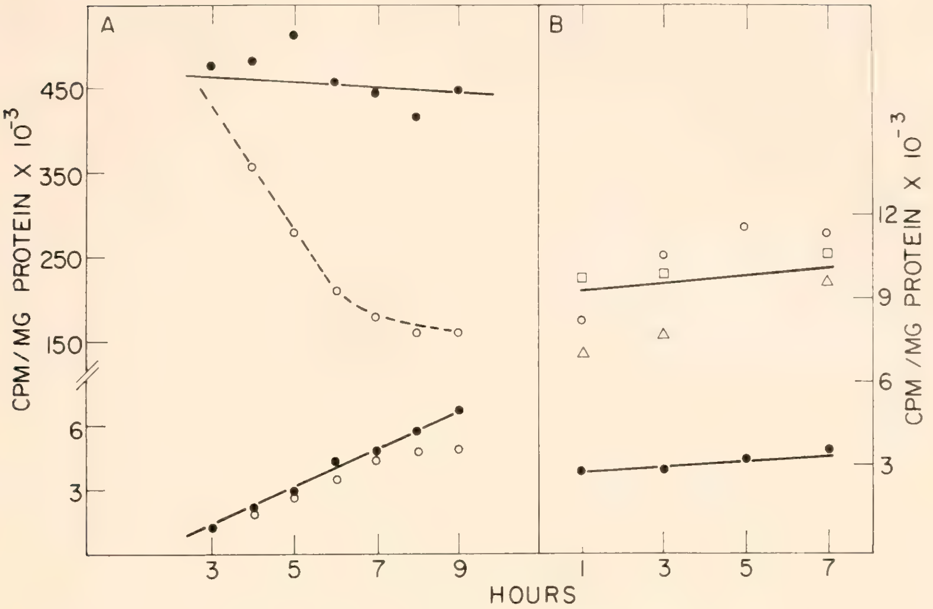

FIGURE 2. (a) Unfertilized eggs of *Strongylocentrotus* were exposed to 1 μc/ml ^{14}C amino acids for three hours, washed, and placed in sea water (●) or sea water containing 0.01 *M* ethionine (○). Both groups incorporated labeled amino acids from their endogenous pools into acid insoluble material (lower curves). The level of uptake (upper curves) remained essentially constant for controls, *i.e.*, the labeled amino acids accumulated during the three hour exposure do not wash off or leak out. Eggs exposed to ethionine lost a substantial portion of their endogenous label during this time. Note that ethionine does not significantly affect the rate of incorporation from the endogenous pool into protein until after four hours of treatment. (b) Eggs were maintained in sea water (●) or sea water containing unlabeled amino acids (methionine □, ethionine ○, or leucine △) at 0.01 M for five hours, washed, and given one hour pulse labels with ^{14}C amino acids at intervals thereafter. The eggs which were exposed to unlabeled amino acids incorporate at a rate 3.0 times higher than controls (overall average). The overall loss of amino acids from pools caused by the amino acid pretreatment, as judged from Figure 2a, is a factor of 2.8. Thus the effect of the amino acid pretreatment seems to be the exchange of the internal pools of many amino acids for the single amino acid present in the medium, lowering the size of pools of all amino acids except that supplied by approximately a factor of three and thereby inflating subsequent incorporation rates by a factor of three. That the value of cpm in the homogenate (uptake) accurately reflects these changes is evidence for its validity as a measure of radioactivity in endogenous amino acid pools.

in the usual way; these were counted with a low background gasflow counter at 11 per cent efficiency or in a Packard Tri-carb scintillation counter at 90 per cent efficiency; a second portion of the homogenate was used for estimation of proteins by the method of Lowry (Lowry, Rosebrough, Farr and Randall, 1951); a third portion of the homogenate was pipetted directly into a scintillation vial and counted in an appropriate medium [either Bray's fluid (Bray, 1960) or a mixture of four parts ethylene glycol monoethyl ether and six parts of the usual toluene based scintillation fluid]. The value obtained in this manner, normalized to amount of protein in the sample, is referred to as "uptake."

The value obtained by the Lowry reaction for protein content of the samples was used to normalize uptake and incorporation value to equal amounts of protein, and therefore presumably to equal numbers of eggs.

RESULTS

Differential response of mature and immature eggs to puromycin

When mature unfertilized eggs are exposed to puromycin for four hours or less (at physiological temperatures), and then placed in puromycin-free water containing ^{14}C amino acids, within three hours they attain a rate of incorporation similar to that observed in untreated controls (Fig. 1). Slightly longer treatment with puromycin is followed by a stimulation of protein synthesis as reported previously (MacKintosh and Bell, 1967). Irreversibility begins to appear only after 6 hours of treatment of unfertilized eggs of *Arbacia*, and after more than 8 hours of treatment of unfertilized eggs of *Strongylocentrotus*.

TABLE II

Incorporation of ^{14}C amino acids by eggs into acid soluble and insoluble material in the presence of unlabeled amino acid in the medium

	Uptake	Acid insoluble	Ratio
control	210,000	2133	0.0100
ethionine	24,000	219	0.0091

Eggs of *Strongylocentrotus* were exposed for one hour to 1 μc/ml ^{14}C amino acids in the presence or absence of 0.01 *M* ethionine. Samples were assayed as usual for radioactivity in the homogenate and for acid insoluble incorporation. Values are per mg protein.

On the other hand, when fertilized eggs are treated with puromycin, the effects become irreversible within one hour of treatment (Ellis, 1966). Similarly, when preparations of unfertilized eggs containing 30 per cent immature eggs (oocytes) are exposed to puromycin for 1.5 hours, protein synthesis in preparations washed free of the antibiotic is greatly depressed. After 3 hours in puromycin-free medium, incorporation in a population of mixed mature and immature eggs is reduced to 35 per cent of controls (Fig. 1b). The capacity of mature unfertilized eggs to recover from puromycin treatment is seen in Figure 1a. The rate of incorporation of precursor into acid insoluble material has exceeded that of controls by 3 hours after washing with puromycin-free sea water.

The effect of concentration and competition on uptake and use of radioactive amino acids

The value obtained by counting radioactivity in the total homogenate is believed to represent uptake of amino acids from the medium as opposed to some type of nonspecific absorption on the following basis:

(1) Eggs which are exposed to ^{14}C amino acids briefly have very low uptake values (Table I).

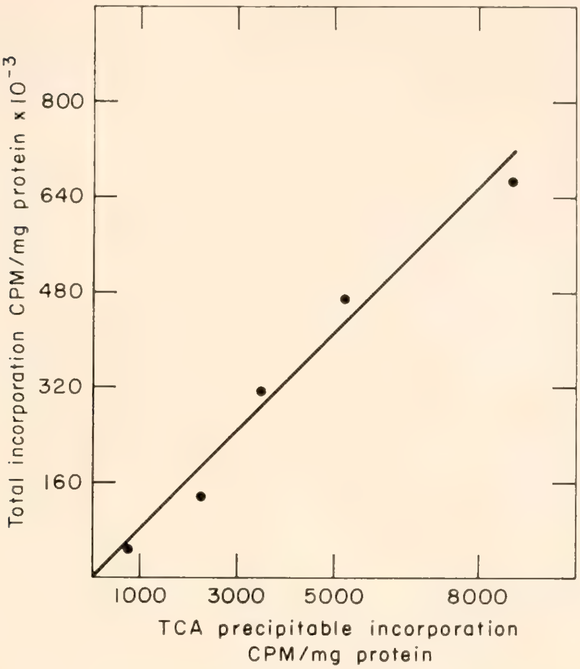

FIGURE 3. *Arbacia* eggs were pulse labeled for one hour with the following concentrations of ^{14}C amino acids: 0.1, 0.2, 1.0, 2.0, 5.0 μc/ml. Eggs were processed as usual and the acid insoluble radioactivity plotted against the total radioactivity in the homogenates. The linearity of the resulting curve shows that incorporation into acid insoluble material is a constant fraction of uptake independent of the actual level of uptake.

(2) Rinsing the eggs does not substantially change the value obtained (Table I).

(3) When eggs are labeled with ^{14}C amino acids, washed and returned to sea water, their uptake (counts in whole homogenate) does not change substantially for several hours (Fig. 2a). (This is also true of eggs exposed to puromycin after labeling.)

(4) If eggs are labeled and washed and placed in sea water containing a single nonradioactive amino acid at 0.01 *M*, their content of radioactivity (uptake) decreases threefold over a period of five hours. Conversely, if the eggs are first exposed to the unlabeled amino acid for five hours and then exposed to labeled amino acids, their rate of uptake is unchanged but their rate of incorporation into acid insoluble material is trebled. See legend, Figure 2b, for our interpretation of this effect. The effect of preincubation with amino acids was also observed by Tyler, Piatigorsky and Ozaki (1966).

(5) If eggs are exposed simultaneously to a single unlabeled amino acid and to the labeled mixture, their uptake is strikingly reduced, and incorporation into acid insoluble material is initially reduced in exact proportion to the reduction in uptake (Table II).

(6) If eggs are exposed to varying concentrations of ^{14}C amino acids uptake is not directly proportional to concentration, but incorporation into acid insoluble material is directly proportional to uptake (Fig. 3).

When unfertilized eggs of the two species were given one hour pulse labels with ^{14}C amino acids over an extended period of time, they displayed an essentially constant rate of protein synthesis (Fig. 4). The incorporation was not only constant on a per milligram protein basis, but was also a constant fraction of amino acid uptake. This constant rate of incorporation was also observed in eggs maintained continuously in ^{14}C amino acids.

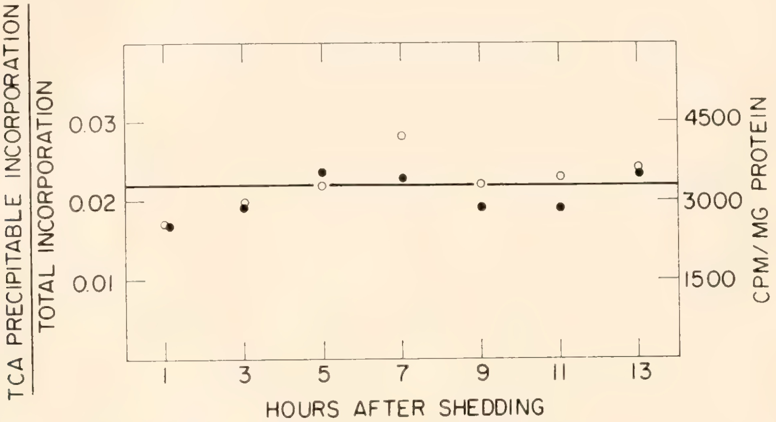

FIGURE 4. Unfertilized eggs of *Strongylocentrotus* were pulse labeled for one hour with 1 μc/ml ^{14}C amino acids at intervals over several hours. Results shown are cpm/mg protein (●) and fraction of uptake incorporated (○).

DISCUSSION

Two types of sea urchin cells which are known to be highly active in protein synthesis [those of embryos (Ellis, 1966) and oocytes (Fig. 1)] have been found to be irreversibly inhibited when returned to puromycin-free sea water after previous exposure to puromycin. In contrast, preparations of mature unfertilized eggs recover to normal rates of protein synthesis after puromycin treatments shorter than 4 hours and do not exhibit signs of irreversible inhibition for at least 6 hours. These observations imply that there is not a detectable fraction of the incorporation of ^{14}C amino acids in preparations of mature unfertilized eggs which can be attributed to either of these classes of highly active cells (oocytes and ovarian fragments, embryos). This finding supports the validity of previously published comparisons of the rate of incorporation in fertilized and unfertilized eggs (Epel, 1967; MacKintosh and Bell, 1967; Bell and MacKintosh, 1967).

A question raised by the foregoing results concerns the basis for the differential response to puromycin which remains to be elucidated.

The experiments on uptake of amino acids and its relation to incorporation of label into proteins establish that surface absorption or other nonspecific uptake is unlikely to be a major factor in this value. The uptake value provides what appears to be a self-consistent measure of radioactivity in endogenous amino acid pools under a wide variety of circumstances. Thus the use of this value in verifying the absence of permeability changes (MacKintosh and Bell, 1967; MacKintosh and Bell, 1969) seems justified.

SUMMARY

Protein synthesis in embryos and oocytes is irreversibly inhibited when the cells are returned to puromycin-free sea water after previous exposure to puromycin. In unfertilized eggs, on the other hand, it is not irreversibly inhibited. This effect and others are used to show that mature unfertilized eggs are actively engaged in protein synthesis, and that direct assessment of the relative rate of protein synthesis in mature eggs is not hindered by contamination with a small population of very active cells.

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CHANGES IN THE EPIDERMAL HISTOLOGY DURING THE SLOUGHING CYCLE IN THE RAT SNAKE *PTYAS KORROS* SCHLEGEL, WITH CORRELATED OBSERVATIONS ON THE THYROID GLAND

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In an initial description of the histological changes in the snake epidermis associated with periodic skin-shedding, Maderson (1965a) established six arbitrary stages to facilitate reference. Stage 1—the resting stage—seen in the post-shedding period, was described as a condition in which a single outer epidermal generation was present above a stratum germinativum. Little if any cell proliferation appeared to occur during this stage, but it was suggested that pronounced changes occurred in the innermost living cells later in the cycle. These layers of cells, which appeared to be present throughout the resting stage, were termed the presumptive lacunar tissue and clear layer. Stages 2 through 5 described the sequential formation and maturation of the component parts of the new inner epidermal generation—*Oberhautchen,* β- and α-layers. In Stage 6, the pre-shedding condition, the inner generation possessed two or three immature cell layers, which were interpreted as representing presumptive lacunar and clear layer cells. Subsequent studies of other lepidosaurian reptiles (Lillywhite and Maderson, 1968; Maderson, 1966, 1967, 1968; Maderson and Licht, 1967) revealed further details of the structure of the epidermal generation and suggested that the original interpretation (Maderson, 1965a) of the pattern of histogenic activity involved in the laying-down and maturation of the lacunar tissue and clear layer was either incorrect, or applicable only to the species *Elaphe taeniura.*

Sembrat and Drzewicki (1936), Goslar (1958), Lynn (1960, 1970), Maderson (1965b) and Maderson, Chiu and Phillips (1970) have reviewed the data suggesting a relationship between the thyroid gland and periodic skin-shedding in squamate reptiles. Although it is generally held that thyroid hormone stimulates shedding frequency in lizards, while inhibiting it in snakes, there has been no attempt to correlate snake epidermal changes with thyroid gland histology comparable to Eggert's (1935) study of *Lacerta.*

The present study of the epidermis and thyroid gland histology of *Ptyas korros* provides additional data on the histogenesis of the snake epidermal generation, correlates thyroid activity with the described cellular changes, and permits some explanation of the previous experimental results suggesting differences between snakes and lizards in this context.

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MATERIALS AND METHODS

From September 19th through October 12th, 1966, 44 mature male *P. korros,* Schlegel were purchased in Hong Kong. Some animals were chosen for purchase on the basis of eye texture to ensure representation of all stages of the sloughing cycle (Maderson, 1965a). Following decapitation, the snakes were weighed, and the weight of the abdominal fat determined. A piece of belly skin was fixed in Bouin's fluid and prepared for histological examination as described by Maderson, (1965a).

The thyroid gland was freed from connective tissue, weighed, and fixed in Bouin's fluid for 48 hrs. It was dehydrated in a series of ethyl alcohol, cleared in chloroform, and embedded in 56° C paraffin. Serial sections through the center of the gland were cut at 7 μ, mounted and stained with hematoxylin and eosin.

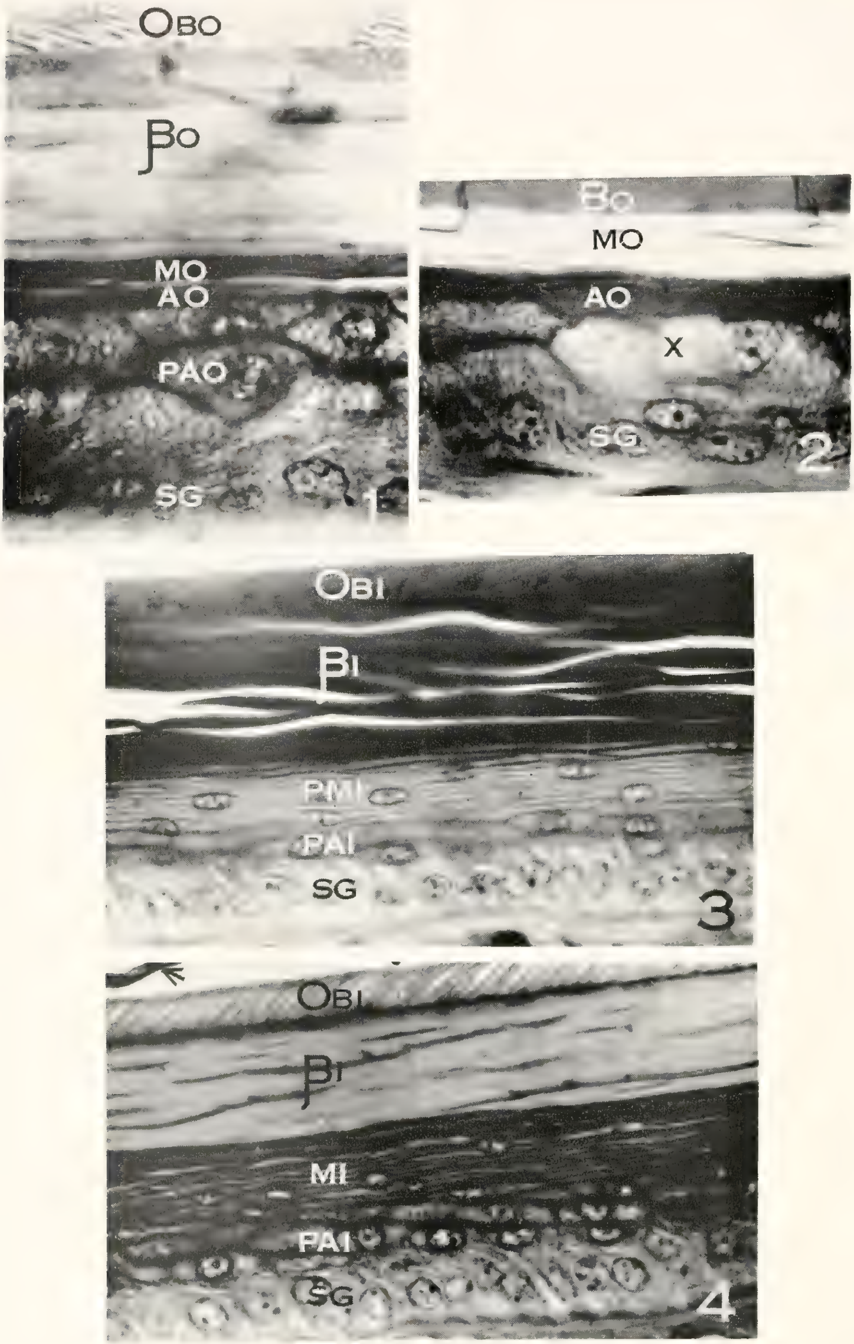
The 44 thyroid glands were divided into 7 groups according to the stage of epidermal development seen in the skin biopsy from the same animal (see Table I). The follicular cell-height for each group was estimated as follows. The 14–31 (mean 20.2) follicles which fell along the longest axis of the gland were examined. The tallest and shortest cell in each follicle was measured with an ocular micrometer. The average of these two values was taken as the cell height for the particular follicle, and the mean of the cell-height for 14–31 (mean 20.2) follicles was taken as the cell height for the gland. The average of the cell heights of all glands examined in any one of the 7 groups was taken as indicating the cell height associated with each of the 7 observable histological conditions of the epidermis.

RESULTS

Epidermal histology

Immediately after shedding, the epidermis of *P. korros* showed an incomplete outer epidermal generation lying above the stratum germinativum (Fig. 1). The generation consisted of a mature *Oberhautchen* (OBO), β-layer (βO) and mesos layer (MO). There was a poorly developed α-layer (AO) and 3–5 layers of presumptive α-cells (PAO). This stage of development is referred to in Table I as Stage 0.

A few days later, the epidermis appeared somewhat different (Fig. 2), and showed a condition referred to in Table I as Stage 1. The α-layer (AO) was then well-developed, and the number of subjacent immature cells reduced by comparison with Stage 0. Stages 2–4 wherein a complete outer epidermal generation (Maderson, 1967) and part of a differentiating inner generation were seen, resembled exactly similar stages described for *Elaphe* (Maderson, 1965a), and these are referred to in Table I. During Stage 5 (Fig. 3), as the new β-cells of the inner generation (βI) finally matured, 8–12 layers of very flattened relatively chromophobic cells were seen just above the columnar, chromophobic cells of the stratum germinativum. These are presumptive mesos cells (see discussion). During Stage 6 (Fig. 4) the β- and mesos cells became completely mature and the nuclei disappeared from them. Presumptive α-cells (PAI, Fig. 4) appeared and began to mature to form a new α-layer. At the time of shedding (Stage 6, Table I), the epidermis consisted of a complete, mature outer epidermal generation which would be lost from the body. The inner generation (which soon becomes the


OBO
βO
MO
AO
PAO
SG
1
βO
MO
AO
X
SG
2
OBI
βI
PMI
PAI
SG
3
OBI
βI
MI
PAI
SG
4

Figures 1–4.

functional body surface) consisted of a mature *Oberhautchen*, β- and mesos layers, a partially mature α-layer and several layers of presumptive α-cells and exactly resembled the picture described for Stage 0 above.

The animals' external appearance during the cycle changed in a slightly different way from *Elaphe* (Maderson, 1965a). Eye cloudiness began in *P. korros* at Stage 4, reached opacity during Stage 5, and cleared while the epidermis showed a Stage 6 histology.

Thyroid activity (*Table I*)

In Table I, thyroid weight in each of the 7 groups is expressed in mg % fat-free body weight. Although some differences are seen, there is no indication that they reflect a cycle of gland activity which can be correlated with the shedding cycle. Thyroid weight and follicular cell height are not correlated. The cell height was greatest around the time of shedding when the epidermis showed either a Stage 6 condition (9.82 μ) or Stage 0 (8.32 μ), and lowest during Stage 3 (5.91 μ). The follicular cell heights during Stages 1, 2, 3, and 4 were significantly lower than during Stage 0 (about 15–30%, $P < 0.05$). During Stages 3 through 6 there was a steady rise in epithelial cell height (about 45%, $P < 0.05$).

Intracellular colloid was seen in the thyroid follicular epithelium in association with all epidermal conditions except Stages 2 and 3. As shown in Table I, the percentage or the number of animals in each instance showing this condition varied; it was typically found in those animals having thyroids with tall follicular epithelia. While the presence of intracellular colloid probably indicated that active

FIGURE 1. The epidermis of *Ptyas korros* seen one day after sloughing (Stage 0, Table I). There is an incomplete outer epidermal generation above a stratus germinativum (SG). The outer generation has mature β- (βO) and mesos (MO) layers. There is a poorly developed α-layer (AO) and three layers of presumptive α-cells (PAO). *Oberhautchen* (OBO). Hematoxylin and eosin. × 850.

FIGURE 2. The epidermis of *Ptyas korros* seen during the "perfect resting stage" (Stage 1, Table I). The β-layer (βO) is out of focus at the top of the picture having separated from the underlying tissues due to splitting of the mesos layer (MO) during histological preparation. The α-layer (AO) is now well developed, and the number of layers of living cells between its base and the stratum germinativum (SG) is reduced by comparison with the condition shown in Figure 1. The cell marked X is probably in the process of final maturation and is the homologue of the "clear cell" shown by Roth and Jones (1967). Hematoxylin and eosin. × 780.

FIGURE 3. The epidermis of *Ptyas korros* showing a very early Stage 5 development. Only the inner generation is shown. Note the characteristic splitting of the cells of the β-layer (βI) in this final stage of maturation (cf. Lillywhite and Maderson, 1968). The mesos cells (PMI) are very flattened units and are quite distinct from the nearly mature β-cells above and first presumptive α-cells (PAI) beneath. Note the columnar shape, chromophobia and oblique orientation of the germinal cells at this time. *Oberhautchen* (OBI). Hematoxylin and eosin. × 540.

FIGURE 4. The epidermis of *Ptyas korros* showing a very early Stage 6 development. Only the inner generation is shown. The β-layer (βI) is mature and resembles the image shown in Figure 1. The mesos cells (MI) have thickened membranes and the first pycnotic nuclei are seen immediately beneath the β-layer. There are now three layers of presumptive α- (PAI) cells. When shedding takes place, the inner generation resembles Figure 1 exactly. Hematoxylin and eosin. × 540.

colloid synthesis was taking place. the significance of the variation at different epidermal stages is unknown.

Many of the thyroid glands of epidermal stages 6 and 0 were highly vascularized, which suggests that these glands were probably actively secreting.

DISCUSSION

Since the initial description of the epidermis of *Elaphe* (Maderson, 1965a), further data on the structure of the squamate epidermal generation have become available (Maderson, 1966, 1967, 1968; Maderson and Licht, 1967; Roth and Jones, 1967). The snake mesos layer was indicated by Maderson (1965a), but was assumed to be the outermost part of the α-layer. Although the relationship of mesos cells to β-cells in terms of protein synthetic capacities is still in doubt, the tissue can now be defined histologically in the snake epidermis. At the onset of Stage 4, presumptive mesos cells are seen as extremely flattened, relatively chromophobic units between the stratum germinativum and the presumptive β-population [Fig. 4 and plate IIa (Maderson, 1965a)]. Their maturation during the latter part of Stage 4 and Stage 5 is accompanied by a chromophobic appearance of the germinal cells [see Fig. 3 in the present study and in Maderson (1965a, pp. 103–106)]. The mesos layer, like the overlying β-layer of the inner epidermal generation is always mature prior to shedding. The mature mesos layer in snake epidermal material is nearly always the site of separation of the β-layer from the subjacent tissues (Maderson and Licht, 1967). The present data from *Ptyas korros* [supported by observations on the cobra (Chiu, Phillips and Maderson, 1969) and

TABLE I

The weight and the cell-height of the thyroid gland, and the percentage of animals with intracellular colloid at different skin stages during the sloughing cycle in Ptyas

Skin stages	Body weight @ (g)	No. of snakes	Thyroid gland ≠		No. of animals with intra-cellular colloid
			Weight mg (%@)	Cell height (μ)	
0	223.82 ± 28.23	11	5.24 ± 0.51	8.32 ± 0.40+	7 (64%)
1	181.20 ± 17.69	5	6.24 ± 1.06	6.78 ± 0.58	1
2	233.67 ± 18.48	6	4.92 ± 0.45	6.78 ± 0.29*	0
3	176.00 ± 16.00	2	5.45 ± 0.92	5.91 ± 0.28	0
4	241.89 ± 29.32	9	6.30 ± 0.64	7.17 ± 0.28	4 (44%)
5	223.33 ± 33.71	6	5.63 ± 0.99	6.90 ± 0.51**	1
6	279.40 ± 18.07	5	7.10 ± 1.87	9.82 ± 1.27	1

≠ Mean ± S.E.

@ Fat-free body weight.

+ Stages 1, 3, and 4 compared with St. 0, $P < 0.05$.

* Stage 2 compared with St. 0, $P < 0.02$.
Stage 2 compared with St. 6, $P < 0.05$.

** Stage 5 compared with St. 4, P = not significant.

several other ophidian species (Chiu and Maderson, unpublished)] compared with the original study of *Elaphe taeniura* (Maderson, 1965a) indicate considerable individual and specific variation in the degree of development of the α-layer at the time of shedding. As indicated previously (Maderson, 1966) *E. taeniura* is unusual in its lack of variation in this respect. These new data on the mesos layer and the pattern of differentiation of the α-layer further substantiate the essential morphological similarity of the epidermal systems in the sub-orders of the squamata.

Stage 1 as described here is the homologue of the "one-cell condition" described for *Gekko* (Maderson, 1966, pp. 46–48) and for *Anolis* (Maderson and Licht, 1967, p. 159). It is believed that this "perfect resting condition" exists throughout a period of time in which no, or at least, very little proliferative activity takes place in the stratum germinativum. Evidence from a variety of experimental studies indicates that it is variation in the duration of this period which provides the basis for different inter-slough periods in different species or individuals. It is therefore probable that this is one of, if not the most important, points where the hormonal milieu could control shedding periodicity (Maderson, Chiu and Phillips, 1970).

The present data indicate that in *Ptyas korros* there is a cycle of changes in thyroid epithelial cell height which can be correlated with the changes in epidermal histology associated with periodic skin-shedding. If one accepts the assumption that when the epithelial cells are high, the gland is synthesizing hormone at an enhanced rate and that this hormone is being released into the blood stream, and conversely decreased height indicates relative inactivity of the gland (pp. 129–130, Pickford and Atz, 1957), we can tentatively draw the following conclusion based on the present results. The gland is very active at the time of shedding, activity decreases throughout the subsequent resting phase, reaching a minimum as the first units of the inner epidermal generation become recognizable and then returns to a high level of activity in the latter part of the renewal phase prior to shedding.

The results of his own studies, as well as those of Schaefer (1933), Krockert (1941) and Halberkann (1953; 1954a, b) led Goslar (1964) to propose the following model for the role of thyroid hormone in the control of the snake sloughing cycle. Goslar suggested that although high levels of circulating thyroxine favored germinal proliferation (he eliminated the "perfect resting condition" and obtained α-hyperplasia by injection with thyroid hormone), generation formation, and subsequent shedding could only occur in the presence of a low level of the hormone in the blood stream. He concluded (Goslar, 1964, p. 4) that the hormone inhibited formation of the "Grenzzone" [the clear layer/*Oberhautchen* complex, the boundary between the outer and inner generations (Maderson, 1967; Maderson and Licht, 1967)]. While this conclusion finds some confirmation in the present results in that the lowest level of thyroid gland activity might be associated with "late" Stage 2–"early" Stage 3 when the clear layer/*Oberhautchen* complex is first recognizable, it may be that the significant factor is the *drop* in thyroid hormone level during the "late" resting phase. Flaxman, Maderson, Szabo and Roth (1968) showed that generation formation is an intrinsic epidermal capacity dependent on an intraepidermal feed-back mechanism. It has been argued elsewhere (Maderson, Chiu and Phillips, 1970) that this mechanism must be *activated* during the late resting phase, otherwise there would not be a Stage 2 or 3 morphological condition to identify. To obtain a more dynamic picture of thyroid hormone involvement with shedding in snakes, information on changes in circulating levels throughout the resting phase is needed.

The results of a directly comparable morphological study of *Lacerta* (Eggert, 1935) are quite contrary to the present data. In *Lacerta,* thyroid activity as judged by histological criteria is low immediately prior to shedding and rises to a maximum immediately afterwards. The level of activity remains high throughout the resting phase, the clear layer/*Oberhautchen* complex differentiates while the level is maximal, and gland activity diminishes during Stages 3–5 to rise again at the time of shedding. In *Gekko gecko* (Chiu and Phillips, 1968; Chiu, unpublished data) there is yet another pattern of thyroid gland activity, showing two peaks of activity during one epidermal cycle. Here, the clear layer/*Oberhautchen* complex differentiates in a rising titer of thyroid hormone, there is a subsequent drop around the time of shedding, followed by another peak 7 days after shedding, so that most of the resting phase is associated with low thyroxine levels.

While it is certain that thyroid gland activity does play an important role in controlling shedding frequency in squamate reptiles, it is probable that other endocrine secretions are equally involved. The presently available data do not permit of the establishment of a model pathway system for either snakes or lizards (see discussion, Maderson, Chiu, and Phillips, 1970). There is no direct information regarding the physiological significance of skin-shedding in squamate reptiles, and indeed there is some doubt as to whether it should be regarded as a consummating event in itself or whether it is merely a side effect of other metabolic activities (Maderson, 1965b; Maderson, Chiu and Phillips, 1970). It is of interest to note therefore that there is good evidence for assuming that it is a more tightly controlled process in some taxa than in others (Salthe and Maderson, 1969). This might indicate that the phenomenon of skin-shedding (in company with factors indirectly associated with it and/or directly controlling it) have been evolutionarily selected for more than once, which could account for known differences in hormonal associations in different taxa.

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SUMMARY

1. Study of the epidermal histology throughout the sloughing cycle in the snake *Ptyas korros* revealed the existence of a well-defined mesos layer and showed that the α-layer is completed in the immediate post-shedding resting-phase. These observations supplement previous histological studies of the snake epidermis and further substantiate the essential homology of the epidermal generation in these forms with similar units in lizards.

2. Thyroid weight is not correlated with follicular cell height nor with epidermal cell changes throughout the sloughing cycle.

3. Changes in follicular cell height, indicative of varying levels of gland activity can be correlated with epidermal changes. The shedding complex and the outermost portions of the new inner epidermal generation differentiate during a period of lowest thyroid gland activity; gland activity is highest around sloughing.

4. These results provide a possible explanation for previous experimental studies of thyroid-sloughing relations in snakes. The problem of the difference between snakes and lizards in this context is discussed.

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CYTOLOGICAL AND IMMUNOLOGICAL INVESTIGATIONS OF SPERM–EGG INTERACTIONS IN SELECTED DECAPODS (CRUSTACEA) AND *LIMULUS POLYPHEMUS* L. (MEROSTOMATA)[1]

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The physiological aspects of sperm-egg interactions have been studied extensively by many investigators (see Metz and Monroy, 1967 for review). These studies include such topics as the effects of egg substances on specificity of attachment, activation of the sperm acrosomal reaction, and release of lytic substances from the acrosome. Many, if not all, of these processes evidently involve gamete surface components; *e.g.*, macromolecules on the surface of the gametes which are believed to interact much like enzyme and substrate or antigen and antibody. Most of this work has been done on echinoderm and amphibian gametes (Metz, 1967 for review).

The present study deals with gamete specificity and the role of gamete surface components, especially antigens, in the fertilization of several representative species of decapod crustaceans and a xiphosuran, *Limulus polyphemus*. The morphology of decapod spermatozoa is quite unusual and bizarre (Brown, 1966) as compared to the so-called typical spermatozoa (*e.g.*, sea urchin sperm; Franklin, 1965). The reptantian spermatozoa used in this study are large and non-motile, possess a number of radiating arms, and have a very large and complex acrosome. Initial sperm-egg attachment is polyspermic. These spermatozoa need to be studied physiologically because their interactions with the egg during fertilization should be compared with those of the typical sperm, upon which most previous physiological information is based. In addition, the large size of the decapod sperm facilitates the analysis of its surface components and their behavior during sperm-egg interactions. Finally, since these spermatozoa are non-motile, one of the usual parameters in fertilization, namely sperm motility, is eliminated and the system is correspondingly simplified.

Materials and Methods

Live specimens were obtained from three sources: (1) the Marine Biological Laboratory Supply Department, Woods Hole, Massachusetts, (2) the Gulf Speci-

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Gametes were obtained from decapods by dissection. Free spermatozoa were obtained from the testes and the seminal receptacle of the female. Spermatozoa contained in spermatophores were obtained from the vas deferens. Mature eggs and oocytes were obtained by maceration of ovarian tissue. Oocytes were considered adequate for experimental use if spermatozoa attached to them in normal proportions (*i.e.,* comparable to the number of spermatozoa attached to normal eggs). Spermatozoa and eggs were obtained from *Limulus polyphemus* males and females by electrical stimulation (Shrank, Shoger, Schechtman and Bishop, 1967).

Antisera were prepared by injecting rabbits subscapularly with an emulsion of whole intact sperm and Freund's complete adjuvant (1:3). Table I presents the kinds of antisera prepared, the species used, and the source of the animals. To assure that equivalent amounts of antibody were used in all experiments, the globulin fraction was separated from the whole serum by precipitating with 18% sodium sulfate (Kekwick, 1940). Protein determinations were made on the globulin fractions using the biuret method (Gornall, Bardawill and David, 1949) and the antibody solutions were then adjusted to equivalent concentrations (25 mg protein per milliliter).

TABLE I

*Types of antisera prepared, the species of animals used and their source**

Antiserum	Antigen prepared from	Species	Source of animals
Anti-*Callinectes* spermatophores	sperm filled spermatophores	*Callinectes sapidus* Rathbun	Gulf Specimen Co.
Anti-*Callinectes* sperm	seminal receptacle sperm	*Callinectes sapidus*	Flor. Mar. Biol. Spec. Co.
Anti-*Callinectes* eggs	eggs	*Callinectes sapidus*	Gulf Specimen Co.
Anti-*Libinia* spermatophores	sperm filled spermatophores	*Libinia emarginata* Leach	Marine Biol. Lab.
Anti-*Pagurus* spermatophores	sperm filled spermatophores	*Pagurus pollicaris* (Say)	Marine Biol. Lab.
Anti-*Homarus* vas deferens	vas deferens	*Homarus americanus* Milne-Edwards	Marine Biol. Lab.
Anti-*Limulus* sperm	sperm	*Limulus polyphemus* Linnaeus	Marine Biol. Lab.

* Other species used in this study (*Cancer irroratus* Say and *Ovalipes ocellatus* (Herbst) were obtained from the Marine Biological Laboratory.

Extracts of sperm and eggs for immunodiffusion experiments were made by homogenizing gametes in a glass homogenizer at 0° C in sea water, 0.85% saline or 0.8 *M* Tris buffer at pH 7.0. In some cases the gametes were treated with 0.5% sodium deoxycholate to enhance membrane disruption. After homogenization the suspension was freeze thawed in an acetone-dry ice bath to aid in the release of antigens from the membranes. In some cases the suspensions were also treated ultrasonically to break up the cell membranes. This consisted of four, 15 second bursts on a Branson sonifier at 0° C. Cellular debris was removed by centrifugation. In most cases the extracts were treated with 0.05% deoxyribonuclease to digest any DNA present which may bind with proteins and precipitate them. Extracts were made up to a ratio of 1:6 tissue to buffer. Antibody concentration was 25 mg protein per milliliter. The gel was prepared from 1% agarose in 0.85% saline with 0.2% sodium azide added as a preservative and 0.05% cadmium chloride added to enhance antigen-antibody precipitate formation.

Some experiments employed papain-digested antibody prepared by the method of Porter (1959) under the following conditions: 100 mg globulin/mg papain (2X crystallized, Sigma Chemical Co.) in 0.1 *M* potassium phosphate, 0.01 *M* cystein, 0.002 *M* disodium EDTA; pH 7.0, 37° C. Digestion was considered complete (usually after 18–22 hours) when the antibody no longer agglutinated homologous sperm in a 2% suspension. At this time iodoacetamide was added to a final concentration of 0.02 *M* to inactivate the enzyme. The digests were then dialyzed exhaustively against sea water.

Table II

Results of cross-insemination experiment reported as the average number of spermatozoa attached per millimeter egg circumference

Spermatozoa	Eggs					
	Libinia	*Callinectes*	*Cancer*	*Ovalipes*	*Homarus*	*Limulus*
Libinia	12.5±3.4*	0.3±0.15	0	0	0	0
Callinectes	0.4±0.22	14.1±3.5	0	0	0	0
Cancer	0	0	43.2±6.5	0	0	0
Ovalipes	0	0	0	8.0±2.2	0	0
Homarus	0	12.8±2.7	9.6±3.3	6.0±1.7	90.6±10.1	0
Limulus	0	0	0	0	0	75.8±9.2

* Standard deviation.

Washed spermatozoa were made up to a 2% suspension. From 10–30 eggs were placed in the well of a spot plate and the excess water removed. Two drops of 2% sperm suspension were added to the well and this mixture was stirred for one minute and then the eggs were washed three times with an excess of sea water. The eggs were then transferred to a microscope slide and observed with phase contrast optics. Ten eggs were chosen randomly and the number of spermatozoa attached to each was determined and recorded. The eggs were not rotated; only the sperm around the egg periphery were counted. The experiment was set up all at one time so that all eggs were treated with the same sperm suspension of each species, *etc.*

Fluorescein-conjugated antibody was prepared by the method of Riggs, Seiwald, Burchhalter, Downs and Metcalf (1958). Conditions for this procedure include 0.15 *M* NaCl, 8% acetone, 0.06 *M* carbonate-bicarbonate buffer (pH 9.0), 10 mg globulin per milliliter, and 0.05 mg fluorescein isothiocyanate (Nutritional Biochemicals Corp., Cleveland, Ohio) per milligram globulin. This mixture was stirred at 4° C for 18 hours. The conjugated protein fraction was isolated by column chromatography (16 × 2.5 cm column) with 0.1 *M* phosphate buffer (pH 7.2) in Sephadex G-25 med. and then dialyzed against sea water for 72 hours. Sperm suspensions (2%) were treated with fluorescein-conjugated antibody for 5 minutes and then washed with sea water. These suspensions were then observed with a Zeiss fluorescence microscope.

RESULTS

Sperm-egg attachment specificity

Five different types of experiments were performed on decapod and *Limulus* gametes. These included cross-insemination, cross-absorption-agglutination, immunodiffusion, antibody inhibition, and antigen localization experiments. These experiments were designed to test for the following: (1) the presence of molecular components essential for fertilization on the surface of sperm and egg, (2) the species specificity of such components, (3) the involvement of such components in sperm-egg attachment, (4) the antigenicity of the components, and (5) the localization of antigenic components with labeled antibody.

TABLE III

Results of cross-absorption-agglutination experiments

Sera		Spermatozoa						
Prepared against sperm	Absorbed with sperm	*Libinia*	*Pagurus*	*Homarus*	*Limulus*	*Ovalipes*	*Cancer*	*Callinectes*
Control serum		0	0	0	0	0	0	0
Libinia	—	+++	0	+	0	0	0	—
	Homarus	+++	—	0	—	—	—	
Pagurus	—	0	+++	++	0	0	0	—
	Homarus	—	0	0	—	—	—	
Homarus	—	0	+	++	0	0	0	—
	Pagurus	—	0	0	—	—	—	
Limulus	—	0	+	0	+++	0	+	—
	Pagurus	—	0	—	++	—	+	
	Cancer	—	0	—	++	—	0	
Callinectes		+	0	+	0	0	+	+++

+ degree of agglutination; 0 = no agglutination; — = no test performed.

Agglutinations were performed with anti-sperm globulin previously prepared against the sperm of each species. The globulin solutions were all diluted to a concentration of 1.6 mg protein per ml, as this was the concentration at which control globulin no longer agglutinated any of the sperm types. One drop of a 2% sperm suspension was mixed together with one drop of anti-sperm globulin on a microscope slide. All tests included two controls: one treated with sea water and one treated with control globulin. Absorptions were performed by adding whole sperm to the globulin and allowing it to stand for three hours at 5° C.

Reciprocal sperm-egg crosses were performed *in vitro* between the following species: *Libinia emarginata, Callinectes sapidus, Ovalipes ocellatus, Cancer irroratus, Homarus americanus,* and *Limulus polyphemus* (Table II). Among the crustaceans employed in these experiments, sperm-egg attachment is polyspermic and most if not all of the attached spermatozoa remain visible at the egg surface for several minutes. Accordingly, comparison of the numbers of spermatozoa attached to the egg surface should provide a measure of sperm-egg affinity. The results of the experiments support this view. Two preliminary qualitative experiments were performed (results not reported). The results of a third experiment are given in Table II as the average number of spermatozoa attached per egg.

All three experiments agree. The results show that with one exception the attachment of gametes of all the species represented are highly self-specific. The spermatozoa of each species attach only to their own eggs. In the one exceptional case *Homarus* spermatozoa attach to the eggs of three other species, *Callinectes*, *Cancer* and *Ovalipes*. These attachments were less firm than normal and the acrosome often was oriented away from the egg surface. *Homarus* eggs failed to cross-attach to the spermatozoa of these three species.

Antigenic specificity

Antibodies were used in cross-absorption-agglutination tests on spermatozoa from the following species: *Libinia emarginata*, *Callinectes sapidus*, *Cancer irroratus*, *Homarus americanus*, *Pagurus pollicaris*, and *Limulus polyphemus* (Table III). Three sets of experiments were performed. All antibodies readily agglutinated their own spermatozoa but none of the antibodies agglutinated the spermatozoa of every species. The agglutinations indicate that antigens are on the surfaces of the spermatozoa. Antigens are present over the entire surface since arm to arm, arm to "head" and "head" to "head" agglutinations occurred. The arm to "head" agglutinations mean that the same antigen appears on the arm and "head." The possibility of arm and "head" specific antigens has not been excluded. The experiments indicate that *Libinia* sperm has at least two surface antigens, one of which is shared with *Homarus* sperm. *Homarus* sperm has at least one antigen which it shares with *Pagurus* sperm. *Limulus* sperm has at least three surface antigens, sharing one of them with *Pagurus* sperm and another with *Cancer* sperm.

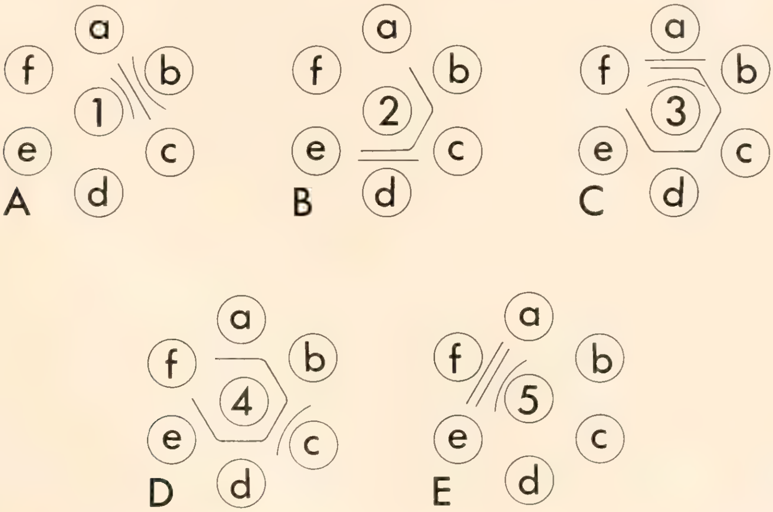

FIGURE 1. Immunodiffusion tests; (1) anti-*Homarus* sperm, (2) anti-*Pagurus* sperm, (3) anti-*Callinectes* sperm (4) anti-*Libinia* sperm, (5) anti-*Limulus* sperm, (a) Callinectes sperm extract, (b) *Homarus* sperm extract, (c) *Libinia* sperm extract, (d) *Pagurus* sperm extract, (e) *Cancer* sperm extract, (f) *Limulus* sperm extract.

This test also indicates that *Pagurus* sperm shares an antigen with *Cancer* sperm. Anti-*Callinectes* sperm serum agglutinates *Libinia*, *Homarus* and *Cancer* spermatozoa. In synopsis, the spermatozoa of *Homarus* has at least one and *Libinia* and *Pagurus* has at least two surface antigens. The sperm of *Limulus* has at least three surface antigens.

Soluble antigens

Immunodiffusion tests (Fig. 1) were performed on extracts of the same species of spermatozoa as used in cross-absorption–agglutination experiments. The extracted antigens could originate from the surface, the interior or both parts of the spermatozoan. A specific antibody was placed in the center well and sperm extracts in the peripheral wells. Precipitin band formation revealed that each species has at least two soluble antigens. *Pagurus* (Fig. 1B), and *Libinia* (Fig. 1D), spermatozoa each have only two antigens. *Homarus* (Fig. 1A), *Limulus* (Fig. 1E) and *Callinectes* (Fig. 1C) spermatozoa have three antigens. The reaction with anti-*Pagurus* sperm globulin indicates that *Pagurus* sperm shares one common antigen with *Homarus* and *Libinia* spermatozoa (Fig. 1B). Likewise, *Callinectes* sperm shares one common antigen with *Homarus, Libinia, Pagurus,* and *Cancer* spermatozoa (Fig. 1C), and *Libinia* sperm shares one common antigen with *Callinectes, Homarus, Pagurus,* and *Cancer* spermatozoa (Fig. 1D).

TABLE IV

Results of inhibition experiments reported as the average number of sperm attached per millimeter of egg circumference

Experiment	Untreated	*Libinia emarginata* spermatozoa treated with:				
		Undigested anti-*Libinia* sperm serum	Digested anti-*Libinia* sperm serum	Undigested control serum	Digested control serum	Digested anti-*Libinia* sperm serum and sheep anti-rabbit globulin serum
1	13.6 ± 3.1	0	0.1 ± 0.10	4.3 ± 1.8	16.3 ± 4.5	—
2	14.9 ± 3.7	0	0.4 ± 0.15	7.9 ± 2.9	5.1 ± 2.1	0.2 ± 0.12

Two drops of a 2% sperm suspension was mixed together with 1 drop of globulin (25 mg protein per ml) in the well of a spot plate. This mixture was agitated for five minutes and then eggs (10–30) were added. After two minutes the excess sperm were washed away and the eggs were examined to determine the numbers of sperm attached. Ten eggs were counted.

Evidence for functional sperm antigens in attachment

Sperm surface antigens can be blocked with specific antibodies to test for their possible role in fertilization. In these experiments *Libinia* spermatozoa were treated with bivalent or univalent anti-sperm globulin (Table IV). The treated spermatozoa were then mixed with eggs in order to determine their capacity for attachment. Bivalent anti-sperm globulin treatment reduced the attachment capacity of the sperm. This result was probably influenced by the fact that whole antibody agglutinates the sperm into large clumps which effectively prevents them from interacting with the egg. However, spermatozoa treated with univalent, non agglutinating anti-sperm globulin also failed to attach to the eggs. Spermatozoa pretreated with digested anti-sperm globulin agglutinate upon subsequent treatment with sheep anti-rabbit globulin (Coombs' or anti-globulin test, Coombs', Mourant and Race, 1945). Treatment of spermatozoa with both whole and digested control globulin did not markedly reduce their ability to attach to the egg.

Localization of sperm antigens by immunofluorescence

Libinia spermatozoa were treated with fluorescein-labeled anti-*Libinia* sperm globulin. Fluorescence was observed over the entire surface of each sperm. No specific area fluoresced more than another. Non-specific staining was checked by treating spermatozoa with fluorescein-labeled control globulin. Such control treated spermatozoa did not fluoresce.

DISCUSSION

The large, non-motile spermatozoa of decapod crustacea should be unusually favorable for studying the initial stages of sperm-egg attachment and interaction. The present study supports this view. It demonstrates species specificity of sperm-egg attachment, specific sperm surface antigens and, at least in *Libinia*, apparent involvement of sperm surface antigens in sperm-egg attachment. Thus the decapods resemble the sea urchin and the few other forms (Tyler, 1946; Metz, Schuel and Bischoff, 1964) that have been studied. The present study extends the analysis to the extent that one well defined process, namely sperm-egg attachment, is inhibited by antibody. No clear cut evidence is yet available to specify the fertilization step or steps inhibited by antisperm sera in other metazoa (*e.g.*, Metz, 1967). It may be argued that the conditions of *in vitro* insemination employed here are sufficiently abnormal to produce excessive and pathological polyspermic sperm-egg interaction. However, Hinsch (in preparation) has examined naturally fertilized eggs of *Libinia* shortly after spawning and finds that these also are highly polyspermic. It will now be of particular interest to determine if the antibody treated spermatozoa can undergo the acrosomal reaction, in other words if the attachment block is at the pre or post acrosomal reaction stage. In the sea urchin the acrosomal reaction is not affected by antibody pretreatment (Fourtner and Metz, 1967).

The cross-absorption agglutination and precipitin tests show that the crustacean spermatozoa have constellations of surface antigens. The distribution and number of these antigens and their solubility can now be extended using additional interspecific combinations and appropriate absorbed sera. Additionally, it may be possible to identify and characterize the "attachment" antigen, using specific antibody as a label. Finally, the interesting question of the antigenic relationship between the surface of the reacted acrosome and the rest of the spermatozoa can probably be investigated readily in this material.

SUMMARY

The initial events of sperm-egg interaction have been examined in several crustaceans. These have large non-motile spermatozoa and sperm-egg attachment is polyspermic.

Sperm-egg attachment *in vitro* is largely species specific among the organisms examined.

Antisperm sera produced in rabbits agglutinates the crustacean spermatozoa. Interspecific agglutinations do occur. Appropriate absorption experiments demonstrate more than one sperm surface antigen.

Spermatozoa pretreated with univalent, non-agglutinating antibody fail to attach to eggs of the species. This indicates that one or more sperm surface antigens are involved in sperm-egg attachment.

Antigens are distributed over the entire sperm surface as shown by the morphology of agglutination and by immunofluorescence.

Two or more soluble antigens can be extracted from crustacean spermatozoa, depending upon species. Some of these antigens give interspecific reactions, others are species specific.

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A GENERAL METHOD FOR THE MONOXENIC CULTIVATION OF THE DAPHNIDAE

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This laboratory has attempted to obtain culture conditions which should satisfy the members of the family Daphnidae that are commonly found in the lakes of the Northeastern United States. We have succeeded with all the commonly available species and have fourteen under monoxenic continuous cultivation, using *Chlamydomonas reinhardii* as the sole food organism.

The classical method for cultivating Cladocera is Banta's stable tea, a pond water extract of horse manure and garden soil (Needham, Gatz and Lutz, 1937). Other successful methods have been developed using mixtures in which either bacteria, protozoa, yeast, or algae are the principal food supply (Mortimer, 1936; Beerstecher, 1952; Murachi and Imai, 1954; Watanabe, Ito and Sasa, 1955; Frank, Bolland and Kelly, 1957; Sasa, Kunieda and Tamiya, 1960; Dewey and Parker, 1964). A monoxenic system was developed for *D. magna* by Treillard (1924) using rabbit erythrocytes and for *Moina macrocopa* by Stuart, McPherson and Cooper (1931) using sterile pond water with suspensions of living bacteria. The individual specimens were freed of microorganisms by repeated washings prior to inoculating the food organism. Fritch (1953) showed that *Chlamydomonas* sp. would support the growth of *D. pulex* if pantothenic acid was added to a system in which bacteria were present. Recently Taub and Dollar (1968) studied the inadequacies of *Chlamydomonas reinhardii* and *Chlorella pyrenoidosa* as food for *D. pulex*.

None of these systems has proved completely satisfactory because of uncontrollable variation in results. Banta (1939) reviewed the problem of "depression" or periods when cultures die out or show a reduced reproduction rate and pointed out that no method was known to prevent the phenomenon. Dewey and Parker (1964) describe the difficulties in obtaining natural water of constant composition free from insecticides and other toxic substances. Anthony D'Agostino and Luigi Provasoli, St. Johns University and Haskins Laboratories (personal communication) have succeeded in devising a dixenic system with *Chlamydomonas reinhardii* and *Scenedesmus obliquus* which will support *D. magna* in continuous culture. The medium contains several salts and vitamins B_{12}, pantothenic acid and thiamin. The method should prevent the aforementioned problems as the cultures can be isolated from variations in food organisms and changes in the composition of the medium.

MATERIALS AND METHODS

Algae. *Chlamydomonas reinhardii* Indiana U. strain #90 and *Scenedesmus obliquus* Indiana U. strain #393 obtained from Dr. Luigi Provasoli were used throughout these experiments as the principal food supply of the *Daphnia*. They

TABLE I

*Composition of the medium (mg/liter)**

	Basic medium	Enriched medium	
		Early formula	Later (improved) formula
Calcium acetate $\cdot$ X H_2O	177.0	59.0	59.0
Potassium penicillin (U.S.P.)	645.0 (10^6 units)	215.0	215.0
Streptomycin sulfate (U.S.P.)	20.0	7.0	7.0
Bovine albumin, fraction V (Armour)	200.0	67.0	67.0
Calcium pantothenate	20.0	7.0	7.0
B_{12}	0.001	0.0003	0.0003
Thiamin	1.0†	0.6	0.6
Riboflavin		0.03	0.4
Nicotinamide		0.3	1.3
Folic acid		3.3	3.3
Biotin		0.3	0.3
Putrescine		0.3	0.3
Choline		0.3	5.0
Inositol		0.6	11.0
Hutner's Trace Elements‡		0.3 ml	0.3 ml

* The medium is made up at three times concentration and frozen in 250 ml amounts. When needed it may be thawed, diluted and filtered through a 2 micron porcelain filter cylinder. Concentrated stock solutions of all the vitamins except biotin, which precipitates, may be stored frozen.

† Only included for a brief time.

‡ Levine and Ebersold's (1958) modification of a trace element mixture designed by Hutner, Provasoli, Schatz and Haskins (1950). We heated the mixture to 100° C and brought it to pH 6 with KOH while hot. The solution is deep green but turns to deep purple on standing. The mixture is stable.

were grown on a yeast extract-acetate medium described by Levine and Ebersold (1958) enriched with one gram per liter of N-Z-Case peptone (Sheffield Chemical Co.) and with agar omitted. The cells were harvested sterilely by low speed centrifugation after 3 or 4 days culture, washed twice in deionized water and added to the *Daphnia* medium (Table I). A delay of 1–2 hours is allowed before the animals are added to allow time for a rise in pH to occur.

Daphnia. The species that are currently under monoxenic cultivation have been obtained from the following sources. *Daphnia magna* from the laboratory of Dr. Luigi Provasoli in bacteria-free continuous dixenic cultivation. *D. pulex* (four strains), 1. Connecticut Valley Biological Supply Co., Southampton, Massachusetts, 2. General Biological Supply House, Chicago, Illinois, 3. Pine Swamp Pond, Connecticut (Lat. 41°53′63N., Long. 73°24′04W.), 4. Candlewood Lake, Connecticut (Lat. 41°28′93N., Long. 73°27′63W.), *D. catawba,* Croton Reservoir, New York (Lat. 41°15′55N., Long. 73°50′54W.), *D. parvula,* Pocantico Lake, New York (Lat. 41°07′06N., Long. 73°50′02W.), *D. retrocurva,* Bantam Lake, Connecticut (Lat. 41°42′66N., Long. 73°13′63W.), *D. ambigua* (two strains), 1. Croton Reservoir, New York, 2. Lake Giles, Pennsylvania (Lat. 41°22′54N., Long. 75°05′90W.), *D. laevis,* Pine Swamp Pond, Connecticut, *D.*

dubia, Candlewood Lake, Connecticut, *D. galeata mendotae* (four strains), 1. Swan Lake, New York (Lat. 41°06'.62N., Long. 73°49'.94W.), (two strains), 2. Candlewood Lake, Connecticut, 3. Croton Reservoir, New York, *Simocephalus serrulatus*, Upper Hadlock Pond, Maine (Lat. 44°19'.19N., Long. 68°17'.28W.), *Scapholeberis mucronata,* Swan Lake, New York, *Ceriodaphnia reticulata*, Dark Entry Forest Pond, Connecticut (Lat. 41°47'.70N., Long. 73°21'.82W.), *C. quadrangula*, Swan Lake, New York, *Moina macrocopa americana*, ephippeal eggs obtained commercially from John and Ruth Fenneberg, P.O. Box 1043, Victorville, California, *M. macrocopa americana* variant, same as above.

Individuals to be isolated are rinsed once or twice and transferred to Falcon 60 × 15 mm "Tissue Culture" petri dishes containing 10 ml of medium with 2×10^6 cells/ml *Chlamydomonas*. Tentative identification is made at this time by stranding the animal on a clean microscope slide. Identifications have been made using Brooks' monograph (1957) for the genus *Daphnia* and Goulden's monograph (1968) for the Moinidae.

TABLE II

The effect of antibiotics and protein on egg development of D. pulex *and survival of young*

	Deionized distilled water	Water with* antibiotics	Water with* protein	Water with* antibiotics and protein
Egg tested	19	9	24	32
Number developing into young	18	4	24	26
Number stuck at interface	9	1	0	0
Survival of young for one day	0	0	16	24
Per cent survival for one day	0%	12%	67%	75%

* Concentration same as in Basic Medium (Table I).

Bacteria-free animals were obtained by the following modifications of the methods of Stuart, McPherson and Cooper (1931) and of Obreshkove and Fraser (1940). As soon as the progenitrix of the strain to be isolated shows well-developed, eyed embryos, it is transferred six or more times through large droplets of sterile medium (Table I) in the hydrophobic top of the plastic petri dish and left for one hour in a dish with 10 ml of medium. It is then rinsed six more times and the brood pouch of the carapace is opened with sterile needles. Care is taken not to express the gut contents during this procedure but doing so does not necessarily mean failure in obtaining bacteria-free young. The embryos in groups of 1–3 are rinsed twelve or more times in droplets of sterile medium on petri dish tops and placed in sterile medium. The important part of this final washing is to use a new sterile capillary pipette for each transfer step and to carry over with the animals as little medium as possible. This procedure usually yields bacteria-free young. If an original culture is especially heavily contaminated the number of rinses may be doubled. Eggs a few hours old may be used as they will develop and hatch but they can be easily damaged by the washing step.

Moina macrocopa must be handled differently. The brood sac is closed in this species and the eggs rupture on release. However, satisfactory results are obtained by washing the young immediately after they are released by the mother.

Bacteriologically sterile animals are transferred twice weekly into new petri dishes with sterile medium and algae and maintained in constant temperature boxes at either 15° C or 20° C constantly illuminated with two 15 watt cool white fluorescent bulbs. They may also be kept in screwtop Falcon 30 ml "Tissue Culture Flasks." Sterility was routinely checked using thioglycollate medium. Anaerobes were ruled out both by darkfield and phase microscopic examination and by the culture method of Schaedler, Dubos and Costello, (1965).

RESULTS

Requirement for embryonic development

Bacteriologically sterile eggs from *D. pulex,* obtained from Connecticut Valley Biological Supply Co., completed embryonic development as well in deionized distilled water as in salt solution. When the young became motile they developed a tendency to stick to the air-water interface. This difficulty was solved by the addition of bovine albumin, fraction V. Table II shows that 0.2 grams per liter accomplished the purpose and that penicillin and streptomycin may be added to the medium to reduce the probability of bacterial contamination.

TABLE III

The effect of various salts tested singly and in pairs on survival of D. pulex *for more than three days (four animals per test)*

	NaCl	KCl	$CaCl_2$	$MgSO_4$	PO_4 buffer
NaCl	0	0	4	0	0
KCl	0	0	4	0	0
$CaCl_2$	4	4	4	4	4
$MgSO_4$	0	0	4	3 (weak)	0
PO_4 buffer	0	0	4	0	0

The salts were dissolved in deionized distilled water with bovine albumin, penicillin and streptomycin as in Basic Medium (Table I) with *Chlamydomonas* and *Scenedesmus* and NaCl 0.004 *M*, $CaCl_2$ 0.001 *M*, $MgSO_4$ 0.0005 *M*, KCl 0.0001 *M*, Na phosphate buffer (pH 6.9) 0.002 *M*.

It had previously been observed that penicillin at 1000 units/ml and that streptomycin at 20 mg/liter were not toxic.

Requirement for juvenile development

The above medium did not support the young even though they were fed a mixture of washed *Chlamydomonas reinhardii* and *Scenedesmus obliquus* whereas a salt mixture allowed continued development. Table III shows that calcium was the necessary factor, for without it, the *D. pulex* died in the next ecdysis. While several calcium salts were tried, calcium acetate was chosen because it is neutral and improves the appearance of the *Chlamydomonas*. Dilutions lower than 10^{-4} molar did not support young of *D. pulex* as can be seen from Table IV. Other salts are brought into the medium with the antibiotics and the algae protoplasm.

TABLE IV

Development from eggs and survival for one week of young of D. pulex *in various concentrations of calcium acetate*

	None added	Concentration of calcium acetate			
		10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Number tested	11	13	14	13	14
Number developing	3	10	7	10	10
Number surviving one week	0	0	0	1	8

Medium consists of bovine albumin, penicillin, and streptomycin as in Basic Medium (Table I) with *Chlamydomonas* and *Scenedesmus* added.

Requirement for fertility

Animals raised to maturity from eggs in the medium as developed to this point, if maintained bacteriologically sterile, survived but produced infertile eggs. These "unproductive" animals constituted the test subjects for the following series of experiments. Table V shows that vitamin B_{12} increased their fertility. However, the effect was temporary and the animals became "unproductive" again. In another experiment shown in Table VI, calcium pantothenate (Fritsch, 1953) was used and

TABLE V

Number of young produced with and without vitamin B_{12}

	Number females tested	Viable young before B_{12} 1st clutch	Viable young after B_{12} 2nd clutch
Control without B_{12}	20	0	10
With B_{12}	19	0	53

Medium consists of calcium acetate, bovine albumin, penicillin, streptomycin as in Basic Medium (Table I) with *Chlamydomonas* and *Scenedesmus*. B_{12} was added immediately after the first clutch.

TABLE VI

Number of viable young and undeveloped eggs produced with and without calcium pantothenate (20 mg/liter)

Medium*	Number females tested	Clutch before pantothenate		Clutch after pantothenate	
		Viable young	Undeveloped eggs	Viable young	Undeveloped eggs
Control without Ca pantothenate	11	2	69	5	40+
With Ca pantothenate	6	1	42	36	0

* Medium consists of calcium acetate, bovine albumin, penicillin, streptomycin, B_{12}, as in Basic Medium (Table I), with *Chlamydomonas* and *Scenedesmus*. Calcium pantothenate was added immediately after the third clutch.

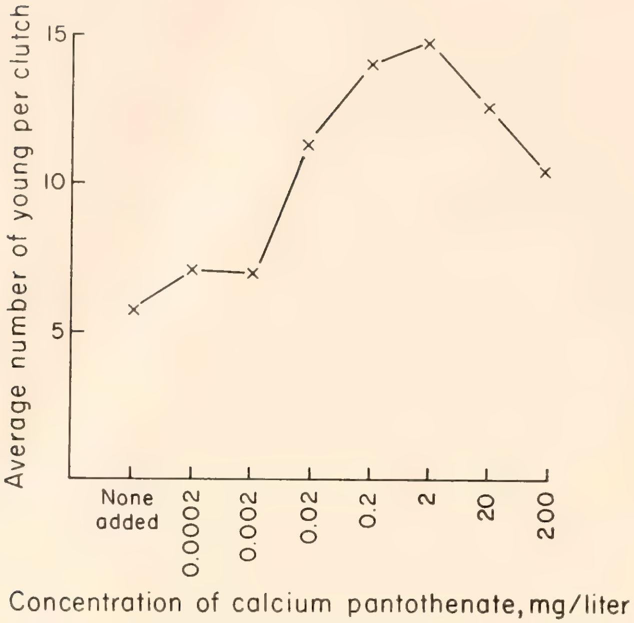

FIGURE 1. Average number of young per clutch in various concentrations of calcium pantothenate (summary of over 400 clutches).

caused a very striking increase in the number of viable young. Raising the amount of vitamin B_{12} 10 or 100 times did not alter the results but leaving it out of the medium produced a sharp reduction in lifespan and in the number of young produced. Calcium pantothenate on the other hand had a definite optimal concentration for production of young (see Fig. 1), but its presence or absence had no effect on the lifespan which averaged 41 days from the day embryological development began. The animals averaged twelve clutches of eggs apiece regardless of the presence or absence of added pantothenate.

Algae requirement

A final experiment in this series was done to find out if it was necessary to add both algae to these cultures. Washed eggs of *D. pulex* were started in basic

TABLE VII

Effect of Chlamydomonas *and* Scenedesmus *on* D. pulex *in basic medium*

	No. females	Average number of young		
		Clutch 1	Clutch 2	Clutch 3
Chlamydomonas alone	15	6.4	9.9	14
Scenedesmus alone	15	0	<1	1.9
Both algae	15	8.4	11.1	18.5

medium (Table I) and fed *Chlamydomonas* alone, *Scenedesmus* alone, or a combination of the two. Table VII shows that cultures with both algae were superior to either alone.

Results with other species

The above basic medium (Table I) permitted us to establish four species of *Daphnia* in bacteria-free continuous cultivation with two algae and another species with one algae. By the time this medium was improved (Table I), we had carried *D. pulex* (Connecticut Valley) 84 generations, *D. pulex* (General Biological) 10 generations, *Scapholeberis mucronata* 63 generations, *Simocephalus serrulatus* 40 generations, *D. magna* 17 generations, and with *Chlamydomonas* alone, *Moina macrocopa* 135 generations. On several occasions these cultures became contaminated and were immediately reestablished in bacteria-free culture by isolating and washing embryos or newborn young. When yeast or fungus occurred as contaminants, daily transfer for a few days was usually all that was necessary to leave it behind.

TABLE VIII

Average number of young on the first brood in three media

	D. pulex Connecticut Valley	*D. pulex* General Biological	*Moina macrocopa**	*Scapholeberis mucronata*	*D. magna*	*Simocephalus serrulatus*	*D. galeata mendotae*
(1.) Basic medium with two algae	4.0 (21)	4.4 (38)	13.3 (80)	2.9 (40)	5.8 (17)	3.2 (20)	0
(2.) Enriched medium with two algae early formula	4.6 (33)	5.6 (52)	15.9 (116)	2.7 (84)	8.8 (18)	4.0 (52)	2.7 (25)
(3.) Enriched medium with *Chlamydomonas* alone, later formula	5.2 (69)	6.0 (76)	17.7 (175)	2.6 (46)	10.1 (51)	3.7 (24)	2.2 (16)
(4.) Repeat of number 3 above	5.8 (28)	6.4 (44)	19.6 (112)	2.8 (41)	10.0 (20)	4.4 (30)	3.1 (38)

* *Moina macrocopa* was always cultured with *Chlamydomonas* alone.

Rows 1, 2 and 3 were run simultaneously. The numbers in parenthesis are the total broods considered in the adjacent average.

Many other species of *Daphnia* were tried in this medium both with and without bacteria with little success. A clone of *D. galeata mendotae* (Swan Lake) became established in a $\frac{1}{3}$ dilution but only when bacteria and a protozoan (*Anisonema*) were present. Passages had to include sufficient old medium to quickly re-establish the contaminants. This indicated that it was likely that the medium was deficient rather than toxic. However, no single factor could be found which would improve the medium enough to support the species *D. galeata mendotae* so we resorted to "shot gun" methods.

Enriched medium, early formula

Immediate success in cultivating *D. galeata mendotae* on algae alone followed adding to the medium a mixture of eight vitamins and Hutner's Trace Elements

(Table I). Table VIII, under the heading "Enriched medium, early formula," demonstrates the effect this medium had on six species of *Daphnia*. It can be seen that *Scapholeberis* may not have done as well but all the others improved as measured by the number of young in their first clutch. Other parameters also showed improvement. They matured slightly sooner, and *D. magna* particularly increased markedly in size and vigor. When tried on other species of *Daphnia* the medium was unsatisfactory. It would support *D. ambigua* and *D. retrocurva* but only when the medium was contaminated with bacteria. It did not support *Ceriodaphnia reticulata*, *C. quadrangula*, *D. parvula*, *D. catawba*, and *D. dubia* even when bacteria were present.

Enriched medium, later formula

At this point a large number of media modifications were tested on the production of young by *D. retrocurva*, *D. galeata mendotae* and *Simocephalus serrulatus* under unsterile conditions using *Chlamydomonas* without *Scenedesmus*. These preliminary experiments indicated that the medium might be improved by increasing the amounts of choline, pyridoxal, inositol, nicotinamide and riboflavin and leaving out *Scenedesmus*. The formulation (Table I) was tested on our seven strains already in bacteria-free serial passage with the type of results shown under "Enriched medium, with *Chlamydomonas* alone, later formula" (Table VIII). The final row shows the results six months later when we had less difficulty with insecticide. It can be seen that each medium modification causes a definite improvement in most strains.

When this final formulation was tried on a wide variety of species of *Daphnia* it was found to support continuous monoxenic cultivation of eight more. No species of the Daphnidae yet tested has failed to reproduce and become established in the presence or absence of bacteria. All the species develop from isolated eggs and are readily obtained bacteria-free. Fourteen species are now under continuous monoxenic cultivation and doing well. They are listed below in order of increasing difficulty found in establishing and maintaining them in the laboratory. The last two have not been tested on anything but the later formula medium with *Chlamydomonas* alone.

Current status of strains under cultivation

Moina macrocopa (two strains) is by far the easiest species to cultivate. It will withstand 0.05 molar salt concentrations, heavy bacterial contamination, and is more resistant to insecticides. It has been maintained bacteria-free for over 200 generations.

Scapholeberis mucronata is also very resistant to insecticides and extremely hardy. The species is small, and while the adults are easy to see because they are deeply pigmented, the newborn young are nearly invisible to the naked eye. It has been maintained for over 200 generations.

D. pulex (four strains) is easy to culture in the laboratory and one strain has been maintained bacteria-free for over 100 generations.

D. magna, although widely used in research, is not by any means as easy to culture as the above. It is also hard to obtain. We have never found it in nature, and although the biological supply houses advertise it, they actually supply *D. pulex*. It is sensitive to insecticide. We have maintained it through 35 and 30 generations. The species was lost once due to insecticide and replenished from a sealed manure-water and algae culture that had supported a small population for one year.

Simocephalus serrulatus is very common in local ponds but compares with *D. magna* in difficulty of maintenance. As soon as the species is obtained bacteria-free it develops the remarkable open-carapace deformity described by Agar (1913). We have not succeeded in preventing this abnormality, but it does not seem to affect their survival and reproduction. It has been maintained for over 80 generations.

D. galeata mendotae is common and easy to maintain in complex medium. Under our conditions either helmeted or unhelmeted animals isolated from nature develop moderately helmeted progeny. It has been maintained for over 30 generations and several strains are under cultivation.

TABLE IX

Comparison of number of young and lifespan of Moina macrocopa *in two media*

	No. animals	No. young		Lifespan	
		Average	Range	Average (days)	Range
Basic medium	37	48.7	7–97	10.6	7–15
	63	34.3	4–88	10.4	3–14
Enriched medium later formula	111	88.4	17–181	11.8	3–20

D. retrocurva is fairly easy to obtain but more difficult to maintain. All the newborn young of some clones immediately become stuck on the air-water interface and must be sunk daily. They remain moderately helmeted and are most sensitive to insecticide. We have maintained it for 18 generations bacteria free.

D. ambigua is a small species, common and easy to cultivate in the more complex medium. It is sensitive to insecticides. It has been maintained for over 40 generations and repeatedly isolated.

D. parvula is similar to *D. ambigua* but requires the later formula medium. It also has been maintained for over 40 generations.

D. dubia is not very common in our experience and has the same characteristics as *D. retrocurva*. We currently have a strain that has gone well for 15 generations and does not get stuck at the interface.

D. catawba is fairly common. We have maintained it for 13 generations but it did not adapt easily to our culture conditions. It is very sensitive to insecticides.

Ceriodaphnia quadrangula compares with *D. ambigua* and *parvula*. It is harder to remove eggs from *Ceriodaphnia* females because of their almost spherical body shape. *Ceriodaphnia* from Swan Lake have large single-spiked fornices in nature and resemble *C. lacustris*, but these spikes are lost on cultivation. The spikes are similar to those described by Rzoska (1956) and Zaret (1969). After 8 generations this species was lost due to insecticide and a new isolate has gone 19 more.

D. laevis was only obtained by us recently, having hatched from ephippeal eggs and has gone through 13 generations. It is a large vigorous animal and probably is among the easier to maintain. Brooks (1957) has identified this as "Banta's *D. longispina*" (page 118) and it is therefore a well known experimental species.

C. reticulata was obtained only recently but has been easy to maintain for 14 generations. As with *C. quadrangula*, the eggs are hard to remove from the female. Older females are preferable because they are much larger than primigravida and have many more eggs.

Effect of medium enrichment on Moina

The effect of the change in medium on the lifespan and total number of young produced by *Moina macrocopa* is shown in Table IX. The later formula medium produces a marked improvement in both parameters particularly if the extremes are considered. Of the 111 animals in the experiment in enriched medium, twenty five or 22% had lifespans of over 15 days. The three experiments reported were done at different times but smaller numbers tested simultaneously show the same general result. We have seen no tendency for the clone of Moina to improve with time alone when the medium is unchanged.

DISCUSSION

The general approach used in this study has been to alternate between modifying the medium to improve the growth of one species and testing the modification on as large a number of species as were currently available. This two-pronged method has the advantage of improving the medium for the species under study and finding new species of *Daphnia* that will grow in it. Working on numerous types has also allowed us to find better species for a given experiment. The best animal to use for testing a modification seems to be one that will survive in unsterile culture, but which, when monoxenic, will not produce more than an occasional fertile egg. If the correct modification is made, this animal will produce viable young almost immediately. In contrast, a species that dies out yields little information and presents problems of obtaining enough animals, while one that produces a few young may, in a better medium, merely produce slightly more young, a less critical endpoint. The assumption underlying this approach is that what is good for one animal is good for another. So far, this has been true in all cases except a clone of *Scapholeberis* which became slightly less productive in the more complex medium. However, it was not necessary to make up a special medium to support this species.

The problem of laboratory contamination with insecticide is most troublesome. If a species is brought into the laboratory and will not survive, the question always arises whether the culture medium is deficient or whether the medium is toxic. Any species that is more sensitive to insecticide than usual will simply seem harder to maintain if the environment is slightly contaminated. The presence of chronic or low level insecticide poisoning is almost impossible to evaluate until sensitivity studies have been made with all the species under cultivation. It is safe to say that all the species reported here have been repeatedly subjected to insecticide and *Moina* and *Scapholeberis* in particular have repeatedly survived levels that have killed off most of the others.

The increase in lifespan and production of young of *Moina* in the enriched medium is remarkable since the strain already was highly productive relative to other species. Even further improvement seems possible since the spread of values for individual animals is very large and the medium has never been modified by direct experimentation with *Moina*. That a relative deficiency in vitamins should have an effect on the production of eggs and young is not surprising but it is not easy to understand why lifespan should be so markedly affected. Vitamin deficiency in higher animals is known to produce specific disease syndromes and may produce death, but an effect on lifespan has not been recognized. There is suggestive evidence that aged humans are "less resistant than the young to the ill effects of restriction of B complex vitamins" (Horwitt, Liebert, Kreisler and Wittman, 1948, page 106) but that is all.

On the other hand, variation in lifespan with diet is a common occurrence in experiments with various species of the Daphnidae. It may be that the biosynthetic mechanism leading toward egg production is capable of drawing so heavily on the reserves of the animal that upon completion of a clutch of eggs, the animal is thrown into pathological deficiency. It is possible that the nutritional state of the parent will also influence the longevity of the young.

The powerful effect vitamins have on the rate of reproduction of Cladocera and other crustacea (Provasoli and Shiraishi, 1959; Shiraishi and Provasoli, 1959; Provasoli and D'Agostino, 1969) may be important in the understanding and control of lake ecology. The evidence of the varying requirements of different species of *Daphnia* may help explain their distribution in nature.

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SUMMARY

Fourteen species of the family Daphnidae have been established under continuous monoxenic cultivation utilizing *Chlamydomonas reinhardii* as sole food organism in a medium consisting of calcium acetate, antibiotics, albumin, trace elements and the water soluble vitamins, folic acid, B_{12}, calcium pantothenate, choline, pyridoxal, inositol, thiamin, nicotinamide, riboflavin, biotin and putrescine. The Daphnidae under cultivation include *Daphnia magna*, *D. pulex*, *D. galeata mendotae*, *D. laevis*, *D. dubia*, *D. retrocurva*, *D. parvula*, *D. ambigua*, *D. catawba*, *Moina macrocopa*, *Scapholeberis mucronata*, *Simocephalus serrulatus*, *Ceriodaphnia reticulata*, and *C. quadrangula*. The requirements for vitamins for some species are more complex than for others. The complete medium is superior for all but *Scapholeberis mucronata* and markedly increases the lifespan and fertility of *Moina macrocopa*.

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REPAIR AND REATTACHMENT IN THE BALANIDAE AS RELATED TO THEIR CEMENTING MECHANISM

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An investigation of the attachment of sessile Cirripedia was initiated to aid future studies on fouling problems. The primary objective was the collection of the barnacle adhesive secretion, generally called the cement, for analysis and tests. The observation that detached or injured barnacles produce a cement-like secretion directed the course of the investigation into a study of the anatomy and functions of the cementing organs to determine the nature of this healing secretion.

The young barnacle begins to grow soon after permanent attachment and metamorphosis have been completed. As is characteristic of the phylum Arthropoda, the body of the barnacle grows in well-defined periods distinguished by the moulting of the exoskeleton. The shell of the barnacle also undergoes cyclic growth (Darwin, 1854) as evidenced by striations on various parts of the shell: the basis, the opercular valves and the conical portion of the shell composed of plates, for which the term "shell wall" is used here, for simplicity. Of particular interest are the striations on the basis, which form concentric circles and permanently record the basis perimeters of previous growth cycles. Darwin (1854) believed that the periods of shell growth are closely related to the moulting cycles. Our histological studies on the development of the cement ducts (Saroyan, Lindner and Dooley, 1968) seem to support the correlation between growth of the basis and moulting. This study shows that the cement duct network is part of a cuticular membrane and is secreted by epithelium cells. At each moulting, this membrane and the duct network are also moulted but become trapped inside the shell. Since each duct network leads to the basis perimeter at that particular time, the relationship between moulting and growth of the basis is strongly indicated. Costlow and Bookhout (1953, 1956, 1957) and Costlow (1956, 1959), conducted extensive studies on the shell growth of *Balanus amphitrite* and *B. improvisus*; and the elaborate experiments designed particularly to explore the connection between shell growth and moulting indicated no direct correlation. They showed that the shell grows continually, though erratically and independently from the moulting (Costlow and Bookhout, 1953, 1956; Costlow 1956, 1959). These findings were based on the outside measurements of the basal perimeter, which also partially include the thickness of the wall plates at the basal edge. Since the perimeter of the basis is overlapped by the edges of the wall, the growth rate of the basis and its relation to the moulting period is not entirely understood.

During growth the leading edges of the calcareous shell grow outward; thus, the basis and the basal margin of the shell walls become larger in diameter and the shell walls are pushed upward. The shell walls and the basis are tightly connected by numerous muscle tissues around the basal perimeter at the inside joints of the leading edges. The contraction of these muscles pull the basis upwards and at the same time the shell wall downwards. Since the basis is usually firmly cemented to the substratum, the wall is pulled down and presses the basal edge of the shell wall tightly down on the substratum. The shell wall rests upon the growing edge of the basis, which is a thin, flexible membrane of chitin, regardless of whether or not the species under examination has a calcareous basis (Costlow, 1956; Newman, Zullo, and Wainwright, 1967). Consequently, this thin flexible rim of the basis is also pressed very tightly to the substratum leaving only a very slight gap. Due to this peripheral pressure, the basis may grow into the recessions and over the irregularities of a solid substratum, faithfully duplicating its surface structure (Pilsbry, 1916; Gregg, 1948) and obtaining maximum contact area for adhesion. The forces produced by the growing barnacle enable it to plow away loose deposits, fouling organisms, and detritus or to dig beneath soft materials, such as clay or certain coatings (Bärenfänger, 1939; Harris, 1946) in order to reach an underlying solid surface. The barnacle cements itself to the substratum by an adhesive which is secreted at the perimeter of the basis and spreads under it to fill any gap between basis and substratum (Darwin, 1854). Due to the peripheral pressure exerted by the barnacle, the gap to be filled and, therefore, the thickness of the cement layer, is normally less than five microns.

In crowded communities, however, the barnacles may develop abnormally. For example, in such communities *Balanus balanoides* grows into elongated shapes, and Darwin (1854, page 147) observed that sometimes only the shell walls of such specimens reach the substratum, while the noncalcareous basal membrane remains suspended and deeply concave. Darwin found that "thickish roots" were hanging from the basal membrane in the resulting gap. He believed these roots to be cement.

This development form apparently escaped the interest of later investigators since only a few references can be found in the literature on this subject. Crisp (1960) describes specimens which survived complete upward displacement by neighboring barnacles but does not mention any adhesive secretion. Crisp also found that specimens of *Balanus balanoides* have some limited mobility under lateral pressure by neighboring barnacles and can be moved along the surface of a smooth substratum several centimeters away from the original point of attachment. He speculated that the advancing edges form new adhesions as the barnacle gradually undergoes lateral displacement. In a recent article (Newman, Zullo, and Wainwright, 1967, page 170) there is a reference to some unpublished observations, "that *Balanus(B) a. amphitrite* Darwin if carefully removed from the substratum without noticeably damaging the wall or calcareous basis, could reattach itself to glass slides, by first cementing and then calcifying itself in place. This is accomplished by protrusion of portions of the mantle from the small spaces that normally occur along the seam between the wall plates and the basis." This short reference, however, neither takes the cementing apparatus into consideration nor goes into detailed explanation of the mechanism of the reattachment process.

The basic anatomy of the cementing apparatus of the Balanidae was described by Darwin (1854). Darwin describes a series of complicated duct networks in a number of species, including *Balanus tintinnabulum*. Each network originates from a pair of pear-shaped vesicles and after much branching and occasionally rejoining the ducts lead to and terminate in orifices around the basal perimeter or, as in the case of older networks, around the concentric circle which marked the earlier growth cycle. The vesicles are also connected by a channel and form a pair of chains resembling strung beads. Darwin believed that the cementing apparatus is a modified part of the reproductive system and that these vesicles are the remains of degenerated cement glands. Not much later, Krohn (1859) recognized the cementing apparatus as a separate organ, and expressed his doubt about the vesicles being secretory glands. Although some additional studies (Pagenstecher, 1863; Gruvel, 1905; and Thomas, 1944) contributed somewhat to the concept of the cementing apparatus of the Cirripedia, no cement glands of any species of the Balanidae were described until quite recently. Lacombe (1966, 1967, 1968) described the cement gland cells and the cementing apparatus of *Balanus tintinnabulum*, although the description of the duct network deviates somewhat from the accurate description of that of the same species by Darwin (1854) and does not mention the vesicles or their function. While Lacombe indicates that each separate duct network of *B. tintinnabulum* develops its own cement gland cells, suggesting a temporary glandular function, in a previous paper (Saroyan, Lindner, and Dooley, 1968), we demonstrated that the gland cells in several other *Balanus* species develop from the cyprid cement glands independently of the duct network; and the same glands function throughout the whole life of the adult barnacle. We also showed that the cement gland cells, although largely intertwined with the ovarian tissues, are more abundant at the lateral area of the mantle, around the end of the two main channels, which rise from the basis into the mantle tissue in close proximity to the lateral scutal depressor muscle. We demonstrated that the cementing apparatus of these *Balanus* species is basically identical to that of the Lepadidae, the other family of Cirripedia, namely in having periodically functioning permanent glands on both sides of the mantle; cement glands consisting of smaller units, which are connected by ducts at a node, where the chemical properties of the secretion are altered; and only one pair of main channels, which conduct the secretion from the node toward the initial attachment point, where the remains of the cyprid antennules can be found. In the Balanidae, a pair of vesicles form around the basal portion of the main channel in each growing period, thus the main channel runs through the vesicles as a continuous tube (Fig. 20). The cement enters the vesicle through the permeable walls of the main channel portion contained within the vesicle. Under normal conditions, the cement does not follow the main channel beyond the newest vesicle toward the initial attachment point and does not reach the older vesicles. At the newest vesicle, which is usually the closest to the basis perimeter, the cement changes its course to find its way through the duct network to the perimeter. As we showed through the histology of their development, the vesicles do not have any secretory function; their purpose is mainly the distribution of the cement into the ducts.

We also found (Saroyan, Lindner, and Dooley, unpublished) that the vesicles may be capable of a pumping action and therefore may be responsible for the transportation of the cement. This pumping action is based upon the barnacle's capability of increasing the pressure inside the mantle cavity by contracting the depressor muscles. As the closed valves depress, they also compress the seawater in the mantle cavity. This pressure increase of the water is transmitted through the resilient walls of the vesicle to its contents as the vesicle is compressed. The soft, flexible walls of the main channel inside the vesicle collapse from the pressure and shut off the passage back in the main channel. The content of the vesicles therefore is forced into the ducts. When the pressure in the mantle cavity is released, the resilient vesicle regains its original shape and volume, drawing more fluid in. By size considerations, there is less resistancy in the main channel than in the ducts, therefore the additional fluid will be drawn into the vesicle from the main channel rather than back from the ducts. With such repeated cycles, the secretion can be forced through the duct network quite rapidly with the vesicle serving as a combination of distributing chamber, pump, and checkvalve.

METHODS

Attached adult *Balanus crenatus, B. glandula,* and *B. cariosus* from Point Reyes, California, and *B. improvisus* from Mare Island Strait, California, were collected on Plexiglas and rubber panels in natural environments. Specimens were removed intact from these panels and successfully reattached on glass microscope slides in the laboratory.

Adult barnacles of *Balanus crenatus, B. glandula,* and *B. improvisus* were also collected on glass microslides in natural environments for microscopic study of the basis and attachment. The specimens were killed, fixed, decalcified, and stained on the original slides. Ten per cent neutral buffered formalin by Lillie (1954) (Pearse, 1961), Baker's (1944) formol-calcium (Pearse, 1961), and Zenker's fixative (Gray, 1954) were used for fixation. Specimens fixed in Baker's formol-calcium for 24 hours, decalcified in Jenkin's fluid (Pearse, 1961) for 24 hours, and refixed in Baker's formol-calcium for 48 hours provided the most satisfactory results.

For studying barnacles in microtome sections, solid paraffin blocks were exposed at the fouling sites. Barnacles attached to the paraffin blocks were allowed to reach sizes up to 6 mm in diameter before they were fixed, then decalcified while still attached to the paraffin substratum. During the later steps of dehydration, the substratum was dissolved to leave the specimen with all substances between its basis and the substratum intact for embedding and sectioning. These specimens were embedded in Paraplast and cut into 10-micron sections.

For general histology, Mallory's Trichrome was adapted but some additional staining techniques, such as Eosin-Aniline blue and PAS, were also used. For the Mallory Trichrome technique, a shortening of the usual staining and rinsing times (Gray, 1954) was necessary to obtain good results with arthropod tissues. This technique consisted of immersing the preparation for one minute in a one per cent Acid Fuchsin solution, one minute in one per cent phosphotungstic acid, and two minutes in a solution containing two per cent Orange G, two per cent oxalic acid, and 0.5 per cent Aniline blue WS. The Acid Fuchsin and the phosphotungstic acid solutions each were followed by a one minute rinse, and the final solution by a five minute rinse in distilled water. The preparation was then dipped briefly in 95 per cent alcohol (15 sec) and then put through two absolute alcohol rinses of three minutes each. Two five minute rinses in two changes of xylene preceded the final mounting in Permount.

RESULTS

During the collection of large specimens of *Balanus nubilus,* one of the barnacles suffered a sizable crack in its basis. After 24 hours, an abundant white, opaque, rubbery exudate, or secondary secretion, was found which filled and sealed this injury (Fig. 1). On numerous occasions it has been observed that barnacles sustain injury such as cracks or breaks in their calcareous bases as a result of the forces produced either by themselves or their neighbors. If vital organs are not seriously damaged or if the injury is not too extensive, the barnacle may survive such an accident by repairing the injuries with these secretions. Microscope preparations show that secretions around or in the cracks of the basis have staining characteristics similar to those of the adhesive cement of the barnacles (Figs. 2 and 3).

Secondary secretions were found not only in injured barnacles, but also in specimens of *Balanus crenatus* and *B. glandula* that were partially or completely separated from the substratum. Some of these separations appear to have been caused by excessive force exerted by the barnacle itself in an effort to press the growing edges of the basis close to the substratum. The pressures created at the perimeter may result in lifting and detaching the central portion of the basis. Such bases become concave and the ensuing gap is usually filled with the secondary secretion (Fig. 4).

Microtome sections of these thick layers of secretions often have a cavernous and vertically striated appearance (Fig. 5). This effect is probably caused by the continuous recession of the basis from the substratum. The gap created between basis and substratum is filled with the fluid secretion, but before hardening can take place, the basis continues to recede. The already viscous secretion may then pull threads of material between the two surfaces, thus creating a loose structure. These new gaps are then filled with fresh secretions during the next period; and so the process is continued until the recession either ceases or continues at so rapid a rate that the secretory system is no longer able to supply enough material to fill the gap.

Another type of separation can occur in gregarious communities where the shell walls of neighboring barnacles may be fused together. Since the shell walls grow up from their bases, a faster growing specimen may lift up and detach a slower growing one from the substratum (Fig. 6). The space between the elevated barnacle and the substratum is usually then filled by the opaque secretion. If the gap is too large to be filled, any secretion present may be seen hanging from the basis, indicating that an effort was made by the barnacle to reach the lost substratum to reattach. The rubbery secretion often covers the whole basis in several millimeter thick layers (Fig. 7) concealing the fine structure of the calcareous basal surface. This fine structure exhibiting the well distinguished cement duct network can be seen on the basis of carefully detached specimens such as the ones used in our reattachment experiments (Fig. 8). These barnacles were removed intact from smooth test panels and subsequently reattached to other smooth surfaces, such as glass microscope slides. Successful reattachments were observed in specimens regardless of whether they were totally submersed in seawater or suspended in such a way that only the opercular region was submersed with the

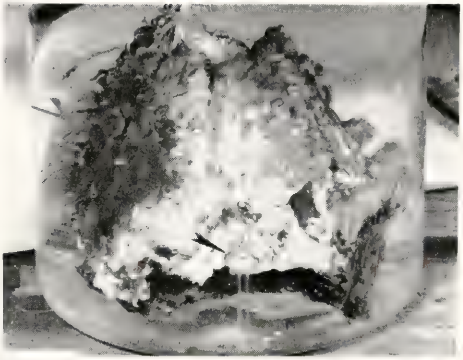

FIG. 1

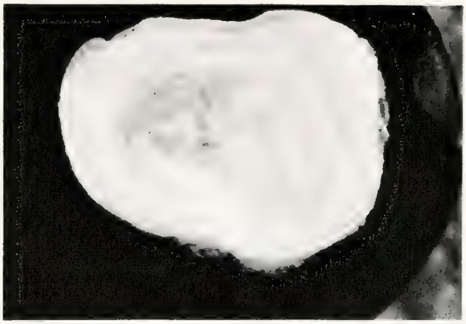

FIG. 4

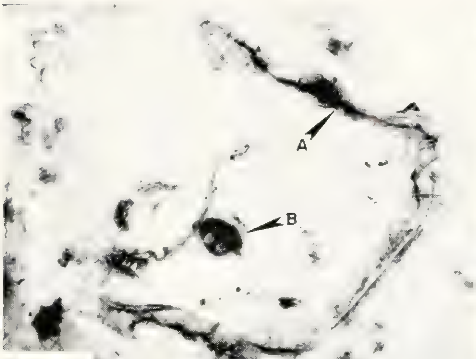

FIG. 2

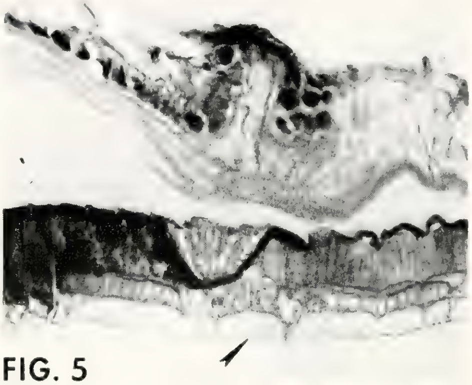

FIG. 5

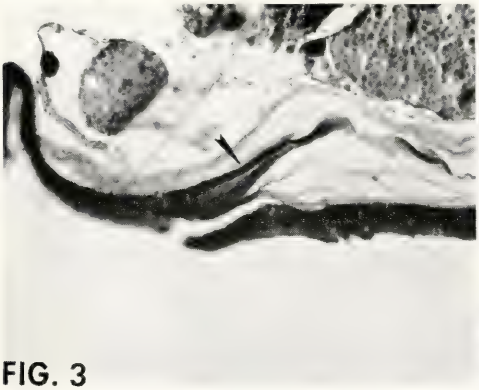

FIG. 3

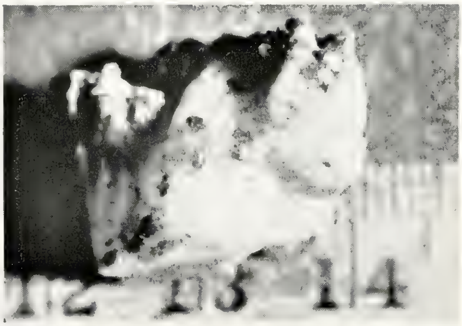

FIG. 6

FIGURE 1. Large specimen of *Balanus nubilus* species suffered a crack in its basis. Within 24 hours, the secretion seen here hardened and repaired the crack.

FIGURE 2. Dark, stained cement in vicinity of (A) circular crack in basis of attached adult barnacle, near (B) initial attachment patch (Wholemount, Masson-Patay, 40 ×).

FIGURE 3. Section through basis of attached adult *Balanus crenatus* showing crack in basis filled with cement (10-μ section, Mallory's Trichrome, 180 ×).

FIGURE 4. Abnormal concave basis on *Balanus crenatus* specimen detached from solid substratum in the laboratory shows abundant secretion, which represents an attempt by the barnacle to fill the central gap between basis and substratum.

basis and the new substratum out of contact with the seawater. The reattachments were accomplished by the white opaque substance secreted on the basal surface and spread between basis and substratum (Fig. 9). The closer the contact of these two surfaces, and hence the thinner the secretion layer, the firmer the reattachment appears to be. The reattachment can be so strong that the shell walls and body will break away before the basis can be detached. If the intervening space between basis and substratum is too large to be filled, thick droplets of secretion appear and hang from the basis (Fig. 10). These reattached specimens can be kept alive indefinitely with proper care and seem to develop normally.

Assuming that the secretion is cement, it could be expected that the secretion would originate from the perimeter of the basis where the newly developed cement duct orifices are located. In general, the secretion does appear at the perimeter, from which it then spreads under the separated or injured area, but centers of secretions can be detected inside the perimeter also (Figs. 9 and 10). In addition, occasionally, an extensive separation or injury occurs isolated within the perimeter and the secretion from the perimeter cannot reach the affected areas in sufficient quantities. Indications are that the barnacle is able to grow new, irregular cement ducts into such a damaged area (Fig. 11). These emergency or secondary cement ducts are larger in diameter than the normal ducts and have few or no bifurcations. Such a duct extends directly from the newest formed vesicle to the damaged area where it ends in an orifice. Normally, only the first vesicle formed after metamorphosis would have such a duct leading from it, as with each succeeding growth period, the duct system becomes increasingly complex in the number of branchings before the final orifices are reached.

The secondary cement ducts usually can be found only in those detached or injured areas where no old primary ducts and orifices can be found as, for example, near the cyprid attachment where the first available duct ends are outside the perimeter of the innermost circle (Fig. 12). There is probably a unique mechanism that enables the barnacle to recognize the need for new ducts and to initiate the growth of these unusual ducts. Likewise, the dissolution of the calcareous matter to permit the growth of new ducts into old sites must somehow occur; similar processes are known in nature.

In the majority of cases, however, where detachment involves basis areas inside the basis perimeter, the reattaching secretion seems to originate from old primary cement duct ends (Fig. 13). This reuse of old ducts is demonstrated in whole-mounts of the basis where several layers of additional secretion lie around the old duct ends (Fig. 14). The layered appearance of the secretion indicates that there was enough time for one layer to harden before the next one was laid down. Since in the course of normal development, the edge of the growing basis is pressed tightly on the substratum and the cement is spread in a very thin layer, these thicker layers of secretion must have appeared subsequent to the growth period during which the primary secretion took place. Therefore, we consider these thicker layers to be secondary secretions or secondary cement.

FIGURE 5. Microtome section through *Balanus crenatus* basis area showing three layers of striated secretions (10-μ section, Mallory's Trichrome, 220 ×).

FIGURE 6. Cluster of *Balanus crenatus* barnacles. Several faster growing specimens have lifted up a slower growing one, which continued to thrive in its elevated position.

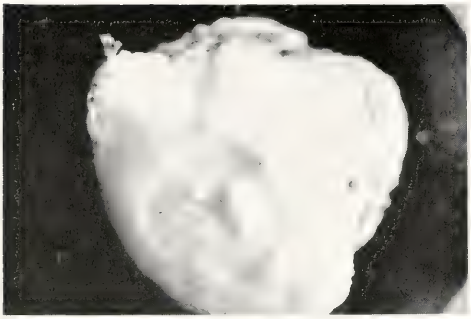

FIG. 7

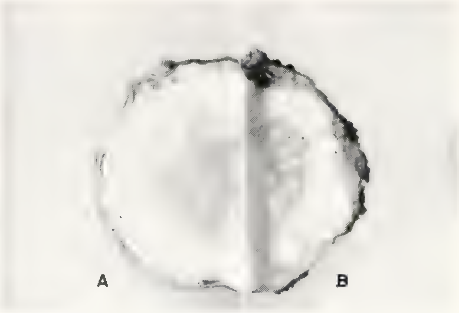

FIG. 10

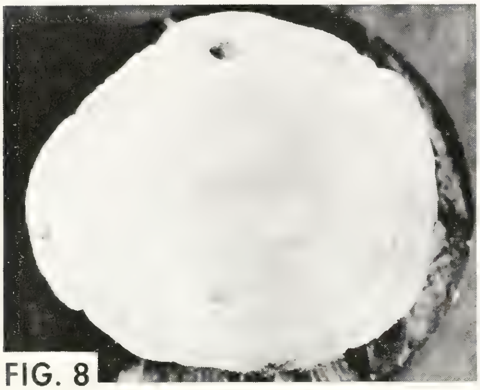

FIG. 8

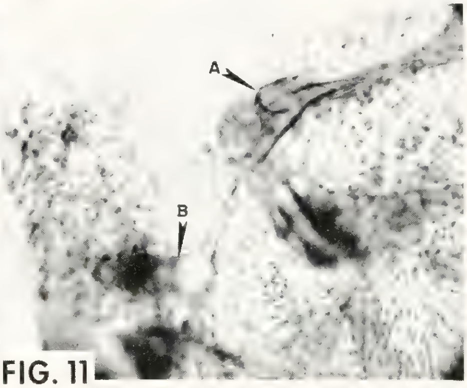

FIG. 11

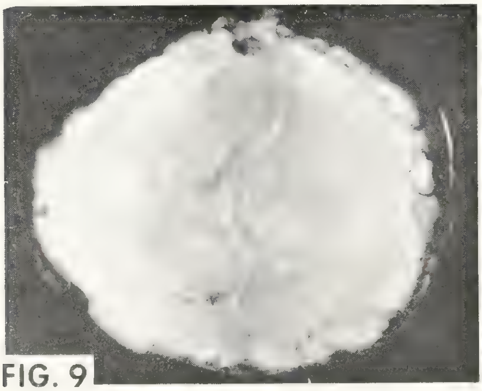

FIG. 9

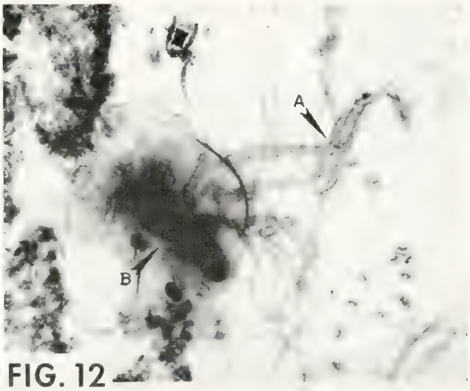

FIG. 12

FIGURE 7. Abundant mass of secretions on basis of *Balanus crenatus* specimen that was naturally detached by other barnacles.

FIGURE 8. Basis of *Balanus crenatus* after removal from smooth test panel in the laboratory.

FIGURE 9. Basis of specimen in Figure 8 after it reattached to a glass microscope slide in the laboratory.

FIGURE 10. Reattached *Balanus crenatus* specimen. Side A is firmly reattached to a glass microscope slide, while side B is left suspended. Drops of secretion hang from the basis on the side where the barnacle was unable to reach and adhere to the substratum.

The quantity of this secondary cement varies according to the size of the animal and the extent of the detachment. It is difficult to estimate the volume of the secondary cement because of its loose, cavernous structure. The volume of the denser primary cement also varies widely according to such factors as the size, age, and growth rate of the barnacle, and the surface characteristics of the substratum. The size and behavior of the very young adult, however, is more uniform. The first adult cement is secreted around the basis perimeter of the first order adult, which metamorphoses from the cyprid. The first order cement ducts are 170 microns long on the average and often not more than two microns in diameter in *Balanus crenatus*. The cement is spread around the perimeter of the basis of the young adult forming an elliptical ring with the following average measurements:

outside semiaxes $A = 3(10^{-2})$ cm
$B = 4(10^{-2})$ cm
inside semiaxes $a = 2.5(10^{-2})$ cm
$b = 3.5(10^{-2})$ cm
thickness $d = 5(10^{-4})$ cm

The average volume of the cement secreted by the first order adult *Balanus crenatus* is:

$$V = \pi\ (AB-ab)d = 5(10^{-7})\ \text{cm}^3$$

DISCUSSION

It was shown that in most cases an injured or detached barnacle uses the old cement duct system for repair or reattachment. This reuse of old ducts is possible only if the duct system connected to these areas is still functional and the passages are still open. Since the ducts were once filled with primary cement, it would seem that when the cement hardens, the ducts would be plugged with solidified material and thereby rendered useless for subsequent secretion. Darwin (1854), however, found that in *Balanus tintinnabulum* the ducts were free of cement. In *Balanus crenatus, B. glandula* and *B. nubilus* we found that this was only partially true. We often observed in these species a few cement ducts and vesicles full of hardened cement among the majority of empty ducts. (Fig. 15). It is impossible to offer any positive explanation for the cement-free status of the ducts without a thorough understanding of the hardening mechanism of the barnacle cement. At this time, however, the available information about the chemistry of the barnacle cement is very limited, therefore, we can resort only to theorizations.

Based on fluid mechanical considerations, it can be demonstrated that perhaps the most important physical requirement for the cement at the time of the secretion is low viscosity. According to the Poiseuille law, the pressure drop of a fluid flowing through a duct is directly proportional to the viscosity and the velocity of

FIGURE 11. New, irregular duct growing directly from a (A) vesicle into a (B) damaged area (Wholemount, TriPARS, 180 ×).

FIGURE 12. Growth of new, irregularly-shaped ducts originating from (A) vesicles into the (B) damaged initial attachment area (Wholemount, TriPARS, 70 ×).

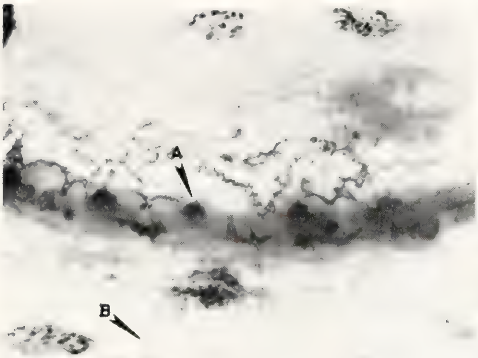

FIG. 13

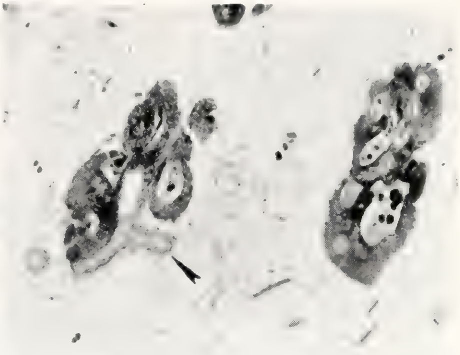

FIG. 16

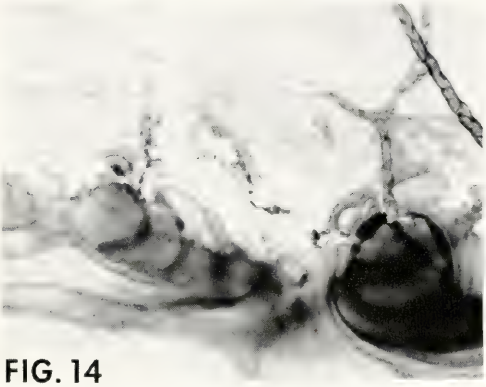

FIG. 14

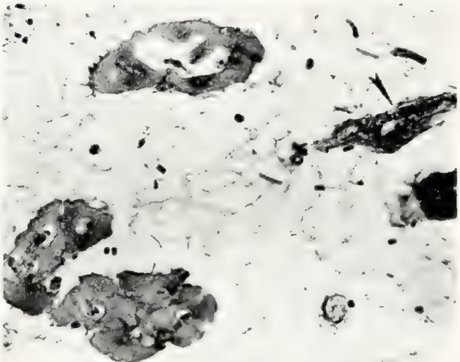

FIG. 17

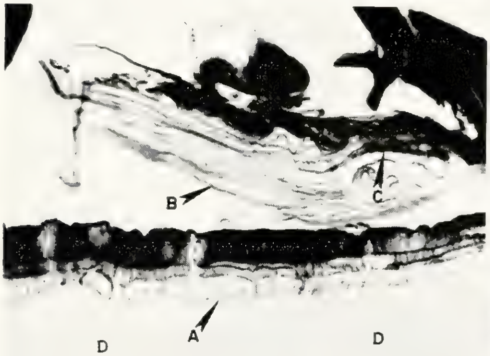

FIG. 15

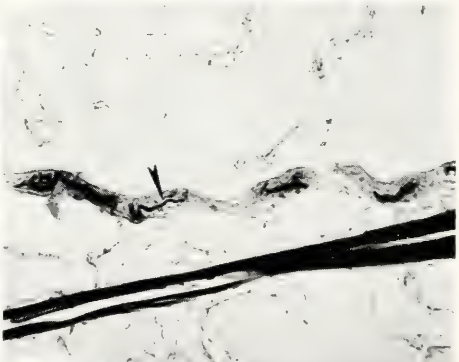

FIG. 18

FIGURE 13. Abundant secondary secretions lie around (A) old duct ends, which now contain hardened cement, at the site of a partial detachment of the basis. Newer (B) ducts, which were unaffected by the accident, are empty (Wholemount, Mallory's Trichrome, 70 ×).

FIGURE 14. Old duct ends with new, thick circular layers of cement around them (Wholemount, TriPARS, 180 ×).

FIGURE 15. Microtome section through basis area of *Balanus crenatus* that was attached to paraffin shows: (A) Thick irregular layers of cement secretions, (B) empty ducts, (C) duct containing cement, and (D) substratum (10-μ section, Mallory's Trichrome, 180 ×).

the fluid, and the length of the duct and inversely proportional to the fourth power of the radius of the duct.

$$\Delta p = \frac{8\eta v l}{\pi r^4}$$

where Δp = difference in pressure at the duct ends, η = absolute viscosity of fluid, v = volume of fluid delivered per unit time, l = length of duct, and r = radius of duct.

It is not yet known how long it takes for the barnacle to secrete the cement, but in a series of yet unpublished experiments, we found already hardened cement within 15 minutes after reattachment. For the sake of the present consideration, permit us to assume that the time interval for the secretion is about one tenth of the hardening time, or $t = 100$ sec. This would mean that the velocity of the secretion or the volume of cement delivered in a second by one of the first order ducts is:

$$v = \frac{\frac{V}{2}}{t} = 2.5(10^{-9}) \text{ cm}^3 \text{ sec}^{-1}$$

therefore, $\Delta p \approx 10^6 \eta$ (dyne cm^{-2}, if η is in poises).

In our example, the numerical value of the additional pressure in dyne cm^{-2} required to deliver the cement at the arbitrarily selected rate through the narrow ducts is about one million times the viscosity of the cement expressed in poises.

If the cement would have a low viscosity near that of water ($\sim 10^{-2}$ poise), the additional pressure would be only: $\Delta p = 10^4$ dyne cm^{-2} (or 0.145 psi). But, if the cement had a viscosity more like glycerol or castor oil (~ 10 poise), $\Delta p = 10^7$ dyne cm^{-2} (or 145 psi).

These considerations strongly suggest that initially the cement is a fluid of low viscosity which solidifies rapidly after secretion. Preliminary analysis of the hardened cement (Lindner and Dooley, 1969) indicates that the cement consists of mainly organic material and is probably a highly cross-linked polymer. The hardening mechanism of the cement therefore is very likely to be due to a polymerization of monomers (Saroyan, Lindner, Dooley and Bleile, 1970). To

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FIGURE 16. The cement precursors are secreted by large, unicellular glands. As more of these glands develop with growth of the barnacle, they are joined to the others by collecting channels, indicated by the arrow, which transport the secretion from each individual gland to the node. The content of the collecting channels does not stain with Mallory's Trichrome (10 μ section, Mallory's Trichrome, 180 ×).

FIGURE 17. The arrow indicates the node where the polymerization is believed to be initiated, because from this point on the channels stain red with Mallory. The red coloration seems to diffuse into the channel from the cytoplasm of the cells composing the node (10 μ section, Mallory's Trichrome, 180 ×).

FIGURE 18. The activated cement, which stains intensely red with Mallory, then passes on through the main channel, shown by the arrow, which carries the cement from its polymerization initiation point to sites of its further distribution (10 μ section, Mallory's Trichrome, 180 ×).

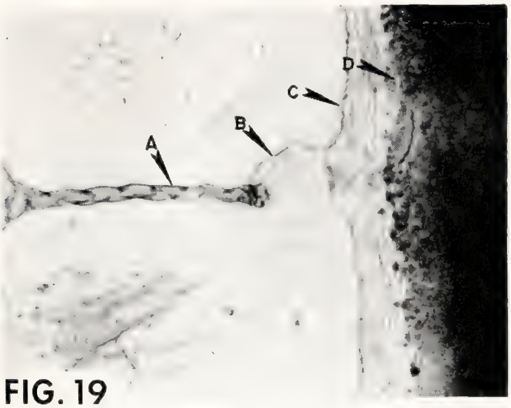

FIG. 19

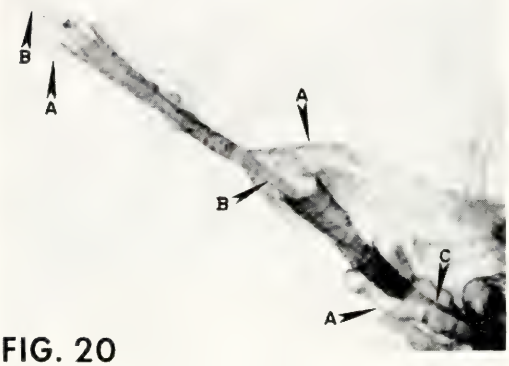

FIG. 20

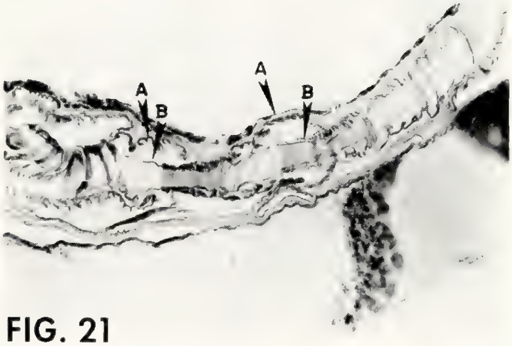

FIG. 21

FIGURE 19. Perimeter of an inner basis circle with (A) empty duct and (B) funnel-shaped orifice leading to the perimeter. The flushing process, following the cement secretion, has washed both the duct area and (C) a circular ring around the edge of the basis free of (D) cement before hardening took place (Wholemount, Mallory's Trichrome, 220 ×).

understand the cementing mechanism it would be of importance to know exactly how and where this polymerization is initiated.

There are indications that the cement polymerization is initiated after the secretion has left the cement glands and before it enters the duct network. Those observations (Saroyan, Lindner, and Dooley, 1968) that substances with staining characteristics similar to that of the hardened cement can be found in the main channel, but never in the secretion collecting side channels, and that the chemical characteristics of the secretion appear to be changed abruptly at the joining node of the collecting channels and the main channel suggest polymerization initiation at the node (Figs. 16, 17, and 18).

Initiation from the environment, namely the seawater, can be ruled out, since it was demonstrated that detached barnacles are also capable of reattachment with their bases and the new substratum out of the seawater. Occasional ducts or vesicles containing hardened cement also make the role of environmental factors questionable, since it is difficult to see how substances from outside could flow back against the cement current through the narrow ducts all the way to the vesicles and mix with the cement to harden it. For the same reason, secretions from the shell walls or elsewhere in the organism, but outside the ducts, ion exchange or pH change outside the orifices are unlikely to play an important role in cement hardening.

Initiation inside the ducts, for instance through the duct walls would not offer sufficient basis to explain the presence of cement-like substances further up in the main channel. Also, portions of secretion passing through shorter or longer ducts would be in contact with the initiating surfaces for a shorter or longer time, thus resulting in unevenly activated cement. It is therefore more likely that the polymerization initiation is restricted to a small portion of the conduits. The node, where the secretion from the scattered cement glands merges into one main channel on each side, is a seemingly ideal site for this.

Using this initiation mechanism, the cement is capable of hardening inside the ducts or vesicles. In order to explain why the majority of the ducts are free of hardened cement, we have to consider some mechanism capable of removing the activated secretion from the ducts either after or before the hardening of the cement is completed.

After the hardening of the cement is completed, the most probable method of its removal would be an enzymatic dissolution or refluidization of the cement, followed by the retention of the altered cement or its absorption through the duct walls. In either case, it is expected that new ducts would be found still containing hardened cement and no cement would be found in the older ducts, since the cement should have been removed from the latter long before. However, we found

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FIGURE 20. (A) Vesicles with (B) main channel passing through them. (C) Collapsed main channel portion acting as a checkvalve to prevent vesicle content from backing up. (Wholemount, Gallego-Garcia, 220 ×).

FIGURE 21. Section through basis of an adult *Balanus crenatus* showing (A) the vesicles and (B) the main channel which passes through them (12-μ section, Mallory's Trichrome, 200 ×).

that all the cement-containing ducts have been older ones, originating in an earlier growing period. Therefore, there is little evidence to support this theory of removal.

Before the hardening is completed, the already activated cement, while it is still in the early stages of polymerization, can be flushed out and replaced by some nonhardening fluid. The most likely flushing fluid may be the non-activated cement containing its monomers but lacking its catalysts, or deactivated cement containing polymerization inhibitors, or modified cement containing non-reacting derivatives of its monomers. The polymerization can be initiated in two general ways, either "spontaneously" or "externally." Spontaneous polymerization is due to an inherent internal property of the system, in which all the necessary optimum conditions are present. External initiation requires the alteration of an existing condition, such as influencing the energetics, reactivity, or charge distribution of the system, or by adding a polymerization agent or subtracting an inhibitor.

Indications are that the polymerization is initiated externally rather than spontaneously. As was pointed out, the hardening seems to be initiated at the node because no trace of substances with cement-like staining characteristics were found in the collecting side channels, which conduct the secretion from the individual gland cells to the node, but from there on, the main channel invariably contains cement-like traces (Saroyan, Lindner, and Dooley, 1968). The polymerization therefore does not seem to be an inherent property of the secretion of the cement gland cells, but rather externally induced at the node by the mixing of different types of secretions from different types of gland cells or from the node itself by any of the above mentioned mechanism.

In view of these considerations, the cementing process starts in the mantle at the cement gland cells with the secretion of the inactive cement precursor, probably a monomer mixture or solution of low viscosity. The secreted precursor is collected by side channels and directed into the node. Here the secretion becomes activated, possibly by mixing with other secreted substances. The activated mixture merges into the main channel which conducts it from the mantle along the lateral scutal depressor muscle to the basis, where it enters the newest vesicle. From this vesicle the cement is distributed throughout the newly developed duct network and orifices around the enlarged perimeter. The cementing period ends with the secretion of the flushing fluid which displaces the still liquid cement from the ducts. In the course of normal development, this non-hardening flushing substance fills all the ducts, forces the cement out beyond the duct orifices and away from the edges of the basis. The cement hardens in this position under the cuticle of the joint connecting basis and shell walls. In this manner, a ten to forty micron wide circular channel is formed between the edge of the basis and the hardened cement, leaving the flushing fluid contained within this seal (Fig. 19).

After the normal cementing period the vesicle ceases to serve as a fluid transporting pump, in conjunction with the main channel, but still acts as an efficient checkvalve that prevents the flushing fluid from backing up and mixing with the new cement. Also, the structure of the vesicle enables it to control the flow of fluids and direct the new cement to where it is needed in the case of injury or detachment.

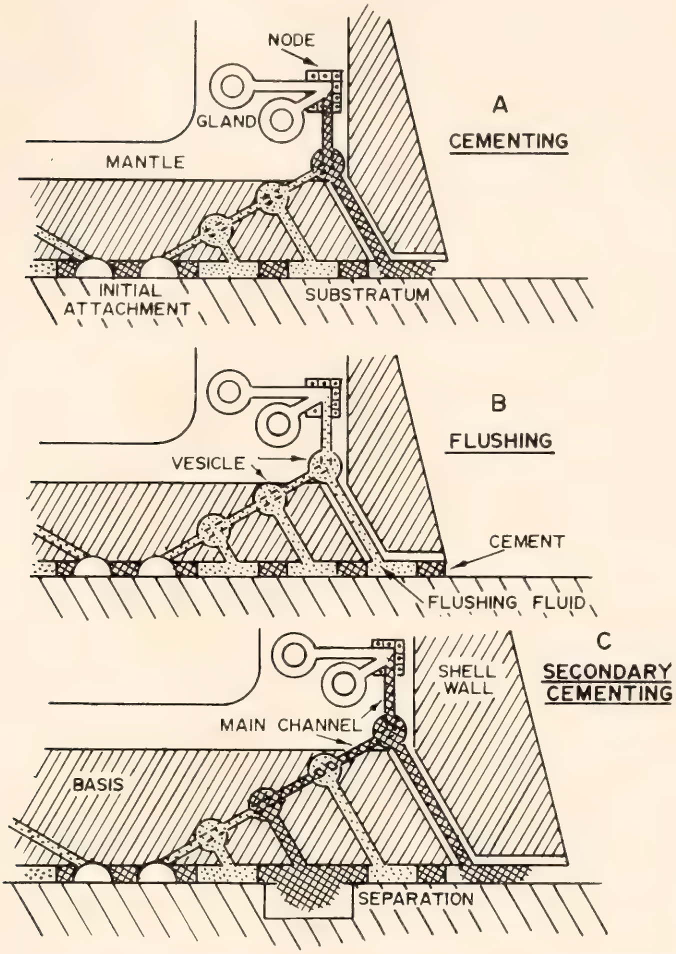
NODE
GLAND
MANTLE
A
CEMENTING
INITIAL
ATTACHMENT
SUBSTRATUM
B
FLUSHING
VESICLE
CEMENT
FLUSHING FLUID
C
SECONDARY
CEMENTING
SHELL
WALL
MAIN CHANNEL
BASIS
SEPARATION

Figure 22. A Secretion of cement from cement gland to newest vesicle and duct network during normal development; B. Flushing of duct network following cement secretion; C. Secretion of cement when a separation from substratum has occurred in the region of an old duct.

As was pointed out, the main channel goes through the vesicles as what appears to be a continuous tube rather than as a simple connection (Figs. 20 and 21). That portion of the main channel which is inside the vesicle is probably permeable, permitting transflux into the vesicle, but largely reducing convection and diffusion between the contents of the main channel and the vesicle. As long as the flushing fluid fills the ducts and vesicles, the system remains in balance and no liquid passes through the permeable walls. In the course of normal development, therefore, the new cement does not go beyond the newest vesicle because the rest of the main channel and duct network is filled with the flushing fluid and no room is available for the cement (Fig. 22A). The new cement can enter only the new vesicle and duct network to be secreted at the perimeter. The still liquid cement is displaced by the non-hardening flushing fluid at the end of the cementing period. The cement hardens outside the orifice and seals the flushing fluid inside the ducts (Fig. 22B).

However, if the basis separates from the substratum, the cement seal of some duct ends breaks and the flushing fluid drains out of the corresponding ducts and vesicles. In the same fashion, a fracture in the basis would sever some ducts and the flushing fluid would also leak out at the injured site. Due to this leakage, the balance of the system is upset. The pressure then drops on the leaking side of the permeable walls, and the loss of flushing fluid makes room for newly secreted cement. The cement is now allowed to pass further down the main channel until it reaches the vesicle affected by the injury and to pass through the permeable membrane of the main channel. The cement enters the vesicle and into the ducts of the injured network and follows the draining flushing fluid to the site of the injury or detachment (Fig. 22C). Since the duct networks of different growing periods are completely isolated from each other except at the vesicles, which are connected only by the main channel, the flushing fluid drains only from duct ends which are affected by the injury and only from those ducts which represent the shortest route between the injury and the corresponding vesicle. Other ducts, unconnected with the injured area, remain filled with the flushing fluid and, hence, the cement bypasses those vesicles which serve the unaffected duct network.

Thus, the vesicle, in conjunction with the main channel not only distributes and transports the cement by means of a pumping action, but also controls and regulates the flow of cement and flushing fluid, as would a valve. The vesicles of different age are situated near each other and are connected by the main channel. This arrangement puts all the vesicles, the corresponding duct networks, and especially the duct ends—regardless of the network to which they belong—almost equidistant from the cement glands. Thus, it is practically as easy to secrete cement through older ducts as through the newest, peripheral duct system. After emergency use, the network is again flushed out and ready for further reuse. In repeated use, however, the flushing process is not always complete because usually larger and irregular amounts of cement are secreted at the emergency locations, thus plugging the orifices. Hence, those reused ducts and vesicles could contain hardened cement.

The authors wish to express their thanks to Dr. Edward Alpen of the Naval Radiological Defense Laboratory for his advice and interest; to Mrs. C. D. Kinney and Mr. Ray Krenik of NRDL, and to the Mare Island Photographic Section of San Francisco Bay Naval Shipyard for outstanding photographic work; and to Mr. Cesar Clavell, Jr., for technical assistance.

SUMMARY

Barnacles, which become partially or totally detached from their substratum in a natural environment, produce a secondary cement secretion. Laboratory experiments demonstrate that the secondary cement can successfully reattach the barnacle to a new substratum. Similar secondary secretion was found at the site

of minor injuries to the barnacle basis. The secondary cement usually has a looser, more cavernous structure than the primary cement, but both secretions have similar staining characteristics.

Microscope preparations indicate that occasionally barnacles are capable of developing new secondary cement ducts leading into the injured or detached areas to secrete secondary cement.

In most cases, however, the existing primary cement duct network is used for the secondary secretion. This is possible only because most of the once used ducts are not plugged by hardened cement, in spite of the fact that the cement can harden inside the ducts. Chemical analysis suggests that the cement is an organic biopolymer and indications are that the cement hardening is initiated inside the organism.

A unique flushing mechanism seems to be responsible for keeping the cement ducts open and ready for reuse. A nonhardening flushing fluid forces the still liquid cement out of the ducts. The cement hardens outside the duct openings sealing the flushing fluid inside the duct network. In case of detachment or injury, the cement seal breaks; the flushing fluid drains out leaving the duct open for the secondary cement secretion.

The vesicles in conjunction with the main channel control the flow of the flushing fluid and the cement. The permeable wall of the main channel portion inside the vesicle reduces the convection and diffusion between the vesicle and the main channel, thus bypassing of vesicles and duct networks not affected by detachment is possible. The wall of the main channel inside the vesicle is also collapsible, thus acting as checkvalve when the vesicle is under pressure and allowing the cement to be pumped only into the ducts toward the secretory orifices.

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THE APPARENT WATER-PERMEABILITY OF *CARCINUS MAENAS* (CRUSTACEA, BRACHYURA, PORTUNIDAE) AS A FUNCTION OF SALINITY

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There is general evidence (Potts and Parry, 1964, pp. 138–143) that the integumental water-permeability of crustaceans inhabiting fresh and brackish water is less than that of marine species, and the adaptive significance of this in reducing the osmotic work-load is obvious. But whether it is possible for an individual crustacean to alter its own water-permeability as an adaptive physiological response to an environmental change of salinity is not self-evident, and conflicting views have been published. Rudy (1967), using tritiated water (T_2O) found adaptive differences in water-permeability among several different species of marine, brackish-water, and freshwater crustaceans, but stated on the basis of his data that the brackish-water, euryhaline, species *Palaemonetes varians* and *Carcinus maenas* could not significantly alter their integumental water permeability. However, I was able to demonstrate on the basis of the uptake of D_2O a significant reduction of water-permeability in response to lowered salinity in the very euryhaline crab *Rhithropanopeus harrisi* (Smith, 1967), and expressed the opinion that Rudy's data on *Carcinus* did not rule out such a response in the latter crab, although Rudy was correct in that his data did not show a statistically significant reduction of permeability. Because *Carcinus* is such an extensively studied animal, it seemed worthwhile to re-examine it by the same D_2O method used to demonstrate a water-permeability change in *Rhithropanopeus* and also in the polychaete worm *Nereis diversicolor* (Smith, 1970), to provide a basis for comparing *Carcinus* with less well known species in other waters.

MATERIALS AND METHODS

Specimens of *Carcinus* of a wide range of sizes and of both sexes were collected in early June on the rocky marine shore near St. Mary's Island, Whitley Bay, Northumberland, in northeastern England. Soft or "paper-shelled" individuals were discarded, as were ovigerous females or crabs lacking one or more claws, or more than a couple of legs. Crabs were maintained at about 15° C at the Dove Marine Laboratory and were adapted for a week or longer in seawater (SW), and in 75%, 50%, 35% and 25% SW, in large plastic boxes provided with stones for shelter. Seawater in these experiments had a chloride concentration of 549 mM/l. Experiments were carried out at the Department of Zoology, University of Newcastle upon Tyne; the adapted crabs needed on a given day were brought to Newcastle in the morning and tested the same day at room temperature (18–19° C).

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Exposure to 5% D_2O in various dilutions of SW followed closely the method used by Smith (1967) for *Rhithropanopeus* except that exposure time was shortened to 15 minutes instead of 30, since *Carcinus* is considerably more permeable to D_2O than the former crab. Seawater was diluted by the addition of local Newcastle pondwater plus sufficient D_2O (90 moles %) to yield 75, 50, 35 and 25% SW. Since the addition of the D_2O to SW caused a dilution to 94% SW, crabs to be tested in this medium were placed in SW diluted to 94% SW with deionized water 2–3 hours before the tests. Crabs above 15 g in weight were tested in a liter of the 5% D_2O solutions, smaller crabs in 500 ml. At the end of each exposure the crab was removed, quickly dried in a cloth towel, and a few drops of blood drawn by puncture of the arthrodial membrane at a leg base. This sample was immediately placed in the large end of a Pasteur pipette, the pipette closed with a small cork, and its tip sealed in a flame. These pipettes, containing samples of blood and medium, were laid on a slide warmer overnight at 50° C to distill the water from the samples into the tips, which projected several inches from the warmer and were cooled by the evaporation of water from a strip of facial tissue paper laid across them with its ends dipping into beakers of water. D_2O contents of blood samples, controls, and the respective media were estimated by comparison with drops of standard solutions (0 to 5% D_2O) in a pair of kerosene/bromobenzene density gradient columns as in Smith's (1967) study of *Rhithropanopeus*. The method is described in detail in Welsh, Smith, and Kammer (1968, pp. 184–188). Values reported are the average of results from the two columns except in a few instances when one subsample was lost or when too little distillate was obtained for two determinations. In a given day's work, half the crabs used had been adapted to and were tested in a higher salinity, half in a lower. This permitted more effective use of the density gradients and helped to randomize differences in time of adaptation and room temperature.

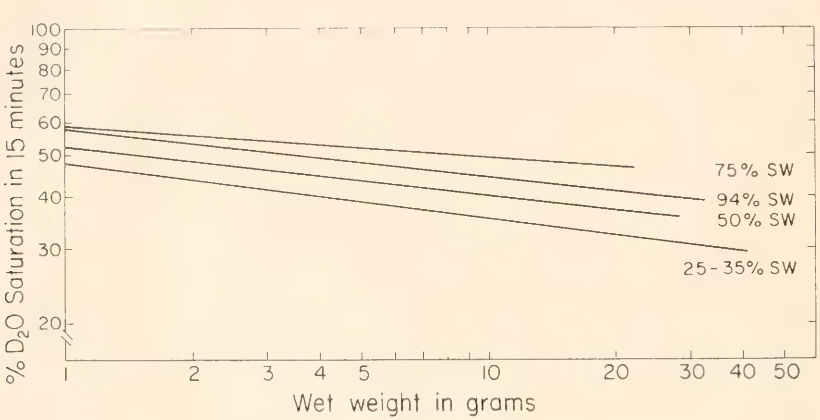

FIGURE 1. Curves relating D_2O-uptake (as % saturation attained in 15 min) to wet weight of *Carcinus* plotted by method of least squares. The equations—Uptake = $a(\text{weight})^{(b-1)}$—are: 94% SW, Uptake = 57.85 $W^{(-0.1152)}$, $n = 23$; 75% SW, Uptake = 58.45 $W^{(-0.0757)}$, $n = 11$; 50% SW, Uptake = 52.07 $W^{(-0.1120)}$, $n = 12$; 25–35% SW, Uptake = 48.37 $W^{(-0.1316)}$, $n = 22$.

Chloride determinations were made on separate samples from 50 of the 68 crabs tested, taken after the D_2O samples by means of disposable capillary pipettes (Drummond "microcaps"), discharged into 1 ml of deionized water before addition of the acid reagent used with the Aminco-Cotlove electrometric chloride titrator.

D_2O-uptake values were recorded as the per cent of the concentration of D_2O in the medium attained in the blood in 15 minutes, taking the D_2O content of the medium as 100%. Since D_2O uptake is weight-specific (Smith, 1967), these "% saturation" values (corrected for controls) were treated following the equation, $S = aW^{(b-1)}$ in which $S = \%$ saturation at 15 minutes, W = wet weight in grams, a = intercept on the ordinate at unit weight, and $(b-1)$ = slope in double log plot. The calculation of (a) and $(b-1)$ was by the method of least squares. For statistical treatment and the calculation of hourly water exchange fractions (K), the values of % saturation were corrected to that of 10 g animals, using the mean $(b-1)$ value of all groups. A weight of 10 g was used rather than unit weight because 10 g was near the mean weight of the animals used, and so reduced possible errors of extrapolation. The hourly water exchange fraction (K) is given by the equation, $K = (2.3/t)\ \mathrm{Log}_{10}\ (100/100-\%\mathrm{Sat.})$, in which K = per cent of body water exchanged per hour (assuming all water is exchangeable), t = time of exposure to D_2O in hours, % Sat. = % concentration of D_2O in water of blood at 15 min, referred to external D_2O concentration as 100%. The data from 25 and 35% SW, being indistinguishable, were pooled and are shown in Figures 1 and 2 as from 30% SW.

TABLE I

Apparent water-permeability of Carcinus *as indicated by per cent D_2O-saturation in 15 min, with the hourly water exchange fractions* (K) *and probability* (t-*test*) *that differences are significant. Values adjusted to body weight of 10 g*

% SW	n	Mean % sat.	Standard deviation	Standard error	K	P
94	23	44.64	±5.48	1.14	2.36	
						> 0.05
75	11	49.54	±5.62	1.69	2.73	
						0.01
50	12	40.44	±4.29	1.24	2.07	
						< 0.025 > 0.01
25–35	22	35.62	±5.32	1.13	1.76	

RESULTS

The influx of D_2O into *Carcinus* is relatively greater in smaller individuals. Curves showing the % saturation of D_2O in the blood as a function of weight after a 15-minute exposure to media containing D_2O are drawn in Figure 1. The slopes of the curves vary from −0.0757 to −0.1316 averaging −0.1088.

The adjustment of individual % saturation values to a body weight of 10 g provides the data shown in Table I and plotted in Figure 2. The specimens of *Carcinus* used in these experiments showed the greatest uptake of D_2O in 75% SW, with a slight reduction ($P > 0.05$) in 94% SW, and a significant reduction of uptake in 50% and 30% SW (*P ca.* 0.01). Calculation of the hourly water exchange fractions (*K*) at each salinity yields values of 2.73 in 75% SW and a low value of 1.76 in 30% SW. Like *Rhithropanopeus*, *Carcinus* reduces its water exchange or its apparent permeability to water at low salinities.

The chloride concentrations of the blood of *Carcinus* in the different salinities of these experiments are shown in Figure 3 and in Table III, line 2. Chloride is strongly hyper-regulated at lower salinities, is isotonic with that of the medium at about 80% SW, and is hypo-regulated in SW. By computations which are explained in the Discussion, the net diffusional (osmotic) influx of water into *Carcinus* at each salinity was calculated (this involved certain assumptions as to the osmotic pressure of the blood) and the results are shown in Table III, line 11. The calculated net water influxes in 50% and 75% SW correspond almost exactly to the volumes of urine produced by *Carcinus* at these salinities, as measured by Shaw (1961) and Binns (1969), shown on lines 13 and 14 of Table III. The calculated net influxes at any salinity are very much higher than those reported by Rudy (1967), but it will be shown in the Discussion that this discrepancy can be largely although not wholly eliminated if the sizes of animals and the temperatures are allowed for. The method of calculating net water influx provides for no net influx of water into *Carcinus* in SW, since the animal is presumed to be iso-osmotic with its medium; hence the present calculated net influxes and those of Rudy fail to account for the production of urine by *Carcinus* in SW, as observed by Shaw and by Binns. Likewise, neither the present results nor those of Rudy after

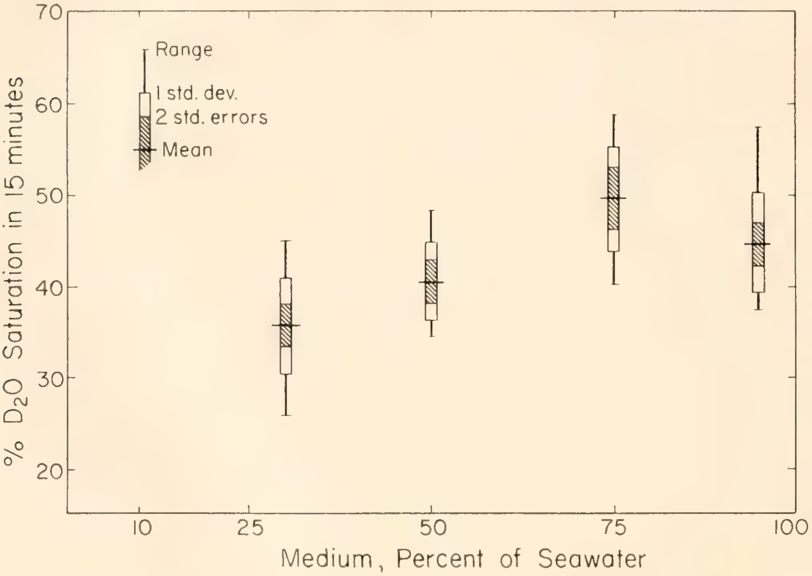

FIGURE 2. Per cent D_2O-saturation attained by *Carcinus* in 15 min as a function of salinity, corrected to a body weight of 10 g (see text). The values in 94% SW are not significantly lower than those in 75% SW ($P > 0.05$), although higher ($P < 0.05$) than those in 50% SW.

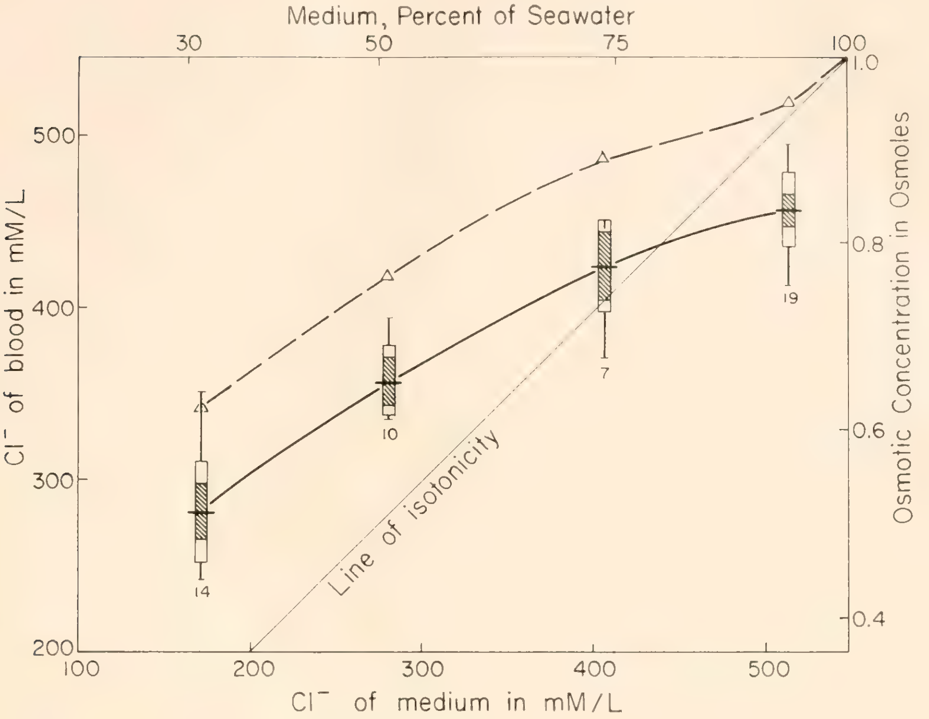

FIGURE 3. Chloride concentration of blood of *Carcinus* (left ordinate) as a function of chloride concentration of medium (bottom, Cl in mM/l; top, % SW). The upper broken curve shows assumed osmotic concentration of blood (right ordinate), as used for calculations in text and Table III. Figures below blocks are numbers of animals sampled.

adjustment for size and temperature (Table III, lines 11 and 12) account fully for the large production of urine in 40% SW measured by Shaw and by Binns (Table III, lines 13 and 14).

DISCUSSION

In discussing the reduced water exchange exhibited by *Carcinus* and *Rhithropanopeus* (Smith, 1967) at low salinities, it should be made clear that an effect and not a mechanism is being described. It is possible that the observed effect is the result of a reduction in the permeability of the integument to water, but in this discussion it is appropriate to use the term "apparent permeability" because no estimate of permeable surface area can be given, and because the effect upon D_2O exchange rate could as well result from a reduction in circulation of blood or irrigation of the gills as from a reduction in cuticular or epidermal water-permeability.

The small reduction in D_2O exchange rate seen in 94% SW is significant only between the 5% and 10% levels of probability, but such an effect is physiologically reasonable and may be expected in certain crustaceans, namely those which show hypo-osmotic regulation at higher salinities. *Carcinus* has not been shown to be a hypo-osmotic regulator, but it may be noted (Fig. 3) that the crabs used in the present study hypo-regulated chloride in SW, and were isotonic in respect to chloride in about 80% SW. Although a measure of osmotic concentration would be more revealing than chloride concentration, this finding, together with the possibly lower apparent permeability in 94% SW, suggests that water-permeability lowering might be looked for under both hyper- and hypo-saline conditions in crabs which are normally exposed to such extremes in nature. Hypo-osmotic regulation has been reported in a number of brackish-water crabs, including some with terrestrial tendencies (Jones, 1941; Gross, 1964), although *Carcinus* is reported to be iso-osmotic or slightly hyperosmotic in SW (Robertson, 1960, and others). Rudy (1967) found that *Carcinus* in his study showed chloride-isotonicity in SW. A clue to the discrepancy between my chloride values and those of Rudy may exist in the finding by Ballard and Abbott (1969) that *Callinectes*, a fully aquatic crab of the same family as *Carcinus* (Portunidae), is hyper-osmotic in SW at 23–24° C, but hypo-osmotic in SW at 28–30° C. Rudy's specimens of *Carcinus* were adapted and tested at 10° C, mine were adapted at *ca.* 15° C and tested at 18° C, hence it is possible that his animals were hyper-osmotic, mine iso-osmotic, in SW. In future studies of *Carcinus* the relationship between adaptational temperature and osmotic concentration of the blood should be critically examined.

In order to compare the present results on *Carcinus* with those of Smith (1967) on the very small *Rhithropanopeus* and with those of Rudy (1967) on larger specimens of *Carcinus*, the earlier data have been recalculated for a body weight of 10 g. In recalculating Rudy's results, the mean $(b-1)$ value of -0.1088 obtained in the present study has been used. The K values so determined are shown in Table II. *Carcinus* in the present study shows higher water exchange fractions (K) than does *Rhithropanopeus*; the K's for the latter (based on D_2O uptake) averaging 39% those of *Carcinus* as indicated by the same isotope. This lower water exchange fraction is consistent with the conclusion that the more euryhaline *Rhithropanopeus* has a lower permeability to water than does *Carcinus*.

When Rudy's data for *Carcinus* (based on T_2O influx at 10° C) are corrected for a weight of 10 g, the K values (Table II) are so far below those of the present study that they have simply been doubled (assuming a Q_{10} of 2) to give values that can be compared with mine obtained at 18–19° C. The Q_{10} of 2 is close to the mean value of 1.90 found by Evans (1969) for water influx in fish. But even with this temperature correction, Rudy's K values for *Carcinus* average only 80% those of the present study.

Are the present data on *Carcinus* (based on D_2O) any more or less reliable than the data of Rudy based on T_2O? Correction of the latter values by reasonable assumptions about weight and temperature leaves a 20% difference in the hourly water exchange fractions, with the K values based on D_2O higher than those based on T_2O. It may be significant that K values for *Palaemonetes varians*, obtained by Rudy (1967) using T_2O, average only 50% of the values obtained on this same species by Parry (1955) using D_2O (Table II). Sufficient data are not available to permit corrections for weight and temperature but, as Rudy remarks, such corrections would probably not account for the whole of the large discrepancy. Obviously needed are water exchange values based on simultaneous T_2O and D_2O uptake measurements on the same species in order to decide whether the K values

TABLE II

Comparisons of hourly water exchange fractions (K) *based on D_2O and T_2O methods:* Rhithropanopeus harrisi, *for weights of 1 g and 10 g, recalculated from Smith (1967);* Carcinus maenas *for weight of 10 g;* Palaemonetes varians *of unspecified weights. See text for rationale of weight and temperature corrections*

Animal	*Rhithropanopeus harrisi* (1 g)		*Rhithropanopeus harrisi* (10 g)		*Carcinus maenas* (10 g)			*Palaemonetes varians* (unspec. weight)	
Isotope	T_2O	D_2O	T_2O	D_2O	T_2O		D_2O	T_2O	D_2O
Reference	Smith unpub.	Smith 1967	Smith unpub.	Smith 1967	Recalculated from Rudy, 1967		This paper	Rudy 1967	Parry 1955
Temp. °C	18–20°	18–20°	18–20°	18–20°	10°	20°	18–19°	10°	?
120% SW								0.64	1.61
94–100% SW		1.55		0.99	0.98	1.96	2.36		
70–75% SW	1.19	1.46	0.71	0.94	0.97	1.94	2.73	0.64	0.95
40–50% SW		1.27		0.85	0.90	1.80	2.07		
25–35% SW		1.05		0.70			1.76		
10% SW	0.75	1.00	0.47	0.67				0.55	
5% SW		0.98		0.65					1.31
1% SW		0.89		0.60					
$\frac{K(T_2O)}{K(D_2O)}$	0.78		0.73		—	0.80		0.50	

yielded by T_2O and D_2O represent an isotope effect or result from individual differences in method. Lacking such a simultaneous experiment with the two isotopes of water, the nearest that can be cited is an unpublished experiment (1968) in which I repeated the 1967 experiments with *Rhithropanopeus,* using T_2O. Without going into detail except that counts were on equal volumes of blood and medium added directly to the scintillation fluid, the results confirmed the change of apparent permeability with salinity in *Rhithropanopeus,* and yielded *K* values averaging 73–78% of the *K* values obtained with D_2O (Table II). This general agreement in the ratio of water exchange values independently arrived at with T_2O and D_2O in the separate experiments on *Rhithropanopeus, Carcinus,* and *Palaemonetes* suggests possibly the presence of an isotope effect causing a faster uptake of D_2O than of T_2O. However, the magnitude of this effect cannot be precisely stated because the recalculation of Rudy's data on *Carcinus* has involved a chain of assumptions.

Despite possible isotope effects, there is no reason to suppose that *relative* water-permeabilities are not fairly represented in comparative studies using either D_2O or T_2O. Rudy's values for the relative water-permeability of the series of crustaceans he studied using T_2O seem valid, and the relative differences in the apparent water-permeability of *Carcinus* as a function of salinity on the basis of D_2O in the present study likewise appear valid. It is suggested, however, that the water influx indicated by D_2O may be somewhat closer to the actual water influx of the animal. In order to test the reasonableness of the water exchange (K) values obtained on *Carcinus*, calculations from these values of net diffusional (osmotic) water influx have been made for comparison with reported values of urine production.

Rudy (1967) calculated the water fluxes of *Carcinus* on the basis of T_2O-exchange fractions (K), and on the assumptions that the osmolarity of SW is 1.0, that the water content of *Carcinus* is 70%, and that the osmolarity of its blood can be represented as % SW based on chloride concentration (in effect, as twice the blood chloride molarity). In the following recalculations I have, in general, followed Rudy's method to facilitate comparison, but have approached the problem of blood osmolarity with somewhat different assumptions. This has been necessary because *Carcinus* in my experiments showed hypotonicity of chloride in SW (Fig. 3) and approximate isotonicity of chloride in 80% SW, whereas Rudy found the chloride concentration of the blood of *Carcinus* in SW to be equal to that of the medium.

Since SW in my experiments had an average chloride concentration of 549 mM/l, its chlorosity is close to 19.1 ‰ (Barnes, 1954) and its freezing point is $-1.88°$ C (Pantin, 1946), corresponding to an osmolarity of 1.01 osmoles. Rudy's assumption of a 1.0 osmolar concentration of SW is thus reasonable, but to apply the same method to blood is not advisable. Nagel (1934) reported that the chloride concentration of the blood of *Carcinus* (as NaCl) accounted for only 88% of the observed freezing-point depression. Lacking a direct measure of osmotic concentration in the animals used in the present study, I have recalculated the osmotic concentration of the blood of *Carcinus* in 94% SW as equivalent to $460 \times (100/88) = 523$ mM NaCl, an increase of 63 mM/l over the chloride concentration of 460 mM/l. The chloride concentration of the blood at lower salinities has then been arbitrarily raised by the same absolute amount, and the osmolarity expressed as % SW (Fig. 3 and Table III, lines 3 and 4). The osmotic concentration of the blood of *Carcinus* in 100% SW has been assumed to be equal to that of SW. By the use of such osmotic concentration values, net water influxes have been calculated by the following steps (data in Tables II and III): *e.g.*, in 30% SW, the mole fraction of water in medium $= 55.56/(55.56 + 0.30) = 0.9946$ (line 5); the mole fraction of water in blood $= 55.56/(55.56 + 0.63) = 0.9888$ (line 6); the mole fraction difference $= 0.0058$ (line 7). This difference accounts for the *net* water influx, which is equal to $0.0058/0.9946 = 0.58\%$ of the total daily influx (line 10). Assuming a 70% by weight water content in *Carcinus* and that all water is exchangeable, the total daily water influx $= K \times 70\% \times 24h = 2957\%$ of body weight in water exchanged per day (line 9). *Net* influx is $0.0058 \times 2957 = 17.2\%$ of body weight per day (line 11). The results of this and similar calculations are shown in Table III, together with the urine volumes of *Carcinus* as

TABLE III

Calculation of daily net water influxes in Carcinus *at different salinities (line 11) for comparison with urine volume estimates of Shaw, 1961 (line 13) and Binns, 1969 (line 14). The net water influx values in line 12 are recalculated from data of Rudy (1967), adjusted for a body weight of 10 g and 20° C. See text for method of computation*

1. Medium, % seawater	30	50	75	94	100
2. Chloride concentration of blood, m*M*/l	282	359	427	460	—
3. Estimated osmotic concentration of blood as m*M* NaCl/l	345	422	490	523	—
4. Adjusted osmotic concentration of blood as % SW	63	77	89	95.3	assume 100
5. Mole fraction water of medium	0.9946	0.9911	0.9867	0.9834	0.9823
6. Mole fraction water of blood	0.9888	0.9863	0.9842	0.9831	0.9823
7. Mole fraction difference	0.0058	0.0048	0.0025	0.0003	0.0000
8. Hourly water exchange fraction (*K*)	1.76	2.07	2.73	2.36	assume 2.36
9. Daily water influx ($K \times 70 \times 24$) as % body weight per day	2957	3478	4586	3965	3965
10. Daily *net* water influx as % of total influx	0.58	0.48	0.25	0.03	nil
11. Daily net influx as % body weight (present data), tested at 18° C	17.2	16.7	11.5	1.19	nil
12. Daily net influx as % body weight (Rudy, 1967), recalculated for 20° C	16.8 (40% SW)	—	9.9	—	nil
13. Daily urine volume as % body weight (Shaw, 1961), 16° C	31.3 (40% SW)	16.5	11.1	—	3.6
14. Daily urine volume as % body weight (Binns, 1969), 9° C	21.1 (40% SW)	16.9	10.8	—	4.4

measured by Shaw (1961) at *ca.* 16° C (line 13) and by Binns (1969) at 9° C (line 14), and net water influxes recalculated from Rudy's data (1967) adjusted to a weight of 10 g and 20° C (line 12).

It does not appear feasible to make a meaningful estimate of the urine volume of *Carcinus* as a function of temperature. If the osmotic concentration of the blood of *Carcinus* behaves as does that of *Callinectes* (Ballard and Abbott, 1969), being inversely related to temperature, then a lowering of water exchange (K) at low temperatures might be to an unknown degree counterbalanced by an increased osmotic gradient favoring a higher net water influx and greater urine volume. Obviously needed are determinations of the osmotic concentrations of the blood at the temperature of the experiment rather than or in addition to chloride determinations, and direct determinations of urine output as a function of temperature.

The net water influxes and urine volumes shown in Table III support the hypothesis that diffusional (osmotic) entry of water accounts for the urine production of *Carcinus* at intermediate salinities (50–70% SW). But if there is no osmotic gradient, *Carcinus* in SW must utilize some form of isotonic water transport. Such transport, in the absence of an osmotic gradient and by the expenditure of metabolic energy, is well known in vertebrates, and several possible mechanisms have been postulated (Diamond, 1965). Any mechanism of isotonic water transport operative in SW might also operate at lower salinities, and so would increase the net water influx above that calculated on the basis of diffusion. Some such mechanism appears necessary in 30–40% SW, where the calculated diffusional net water influx is well below the reported urine production (Table III). Alternatively, one might consider a system such as that suggested by Ussing (1954), in which inner diffusional areas are in series with outer pore-like spaces such that, once a diffusional net influx is established, a bulk flow of water is set up in the "pores" of sufficient velocity to counteract or reduce diffusion in the opposite, outward, direction. Such a system would have the properties of a rectifier or one-way valve, admitting water but restricting the outward diffusion of water below what would be expected on the basis of the water concentration in the blood. The water thus prevented from diffusing out would be available for disposal in the urine, over and above the net diffusional influx calculated from the mole fraction difference between water concentrations in blood and medium. The diffusional influx would be as expected; what would be reduced is the diffusional efflux.

It is evident that the water economy of *Carcinus* is still incompletely understood. Future work could profitably be directed to several problems. Is the apparent reduction of water-permeability with salinity the result of a reduction of circulation, either in the medium bathing gills or gut or in the circulation of the blood, or is it the result of an actual change of integumental permeability? The possibility that permeability reduction may accompany either hyper- or hypo-osmotic regulation seems physiologically adaptive and reasonable, but more critical examination is needed. This should be studied in some crab normally exposed to seasonal hypersaline conditions (Gross, 1961). The problems of correlating the water-permeability figures reported by different workers using T_2O and D_2O might be solved by simultaneous double-tracer studies of water influx in order to reduce individual operational variation and to evaluate possible isotope effects. In all such work the weights of the animals used must be taken into account and temperature controlled, since hourly water exchange fraction (K) varies with body weight as well as with salinity and temperature, and is thus no more a constant for a species than is respiratory rate.

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SUMMARY

1. The apparent water-permeability of *Carcinus maenas*, as measured by D_2O influx, is 2–3 times higher than that of the more euryhaline crab *Rhithropanopeus*.

2. Like *Rhithropanopeus*, *Carcinus* shows a reduction of water-exchange rate at lower salinities. The highest hourly water-exchange fraction is in 75% SW ($K = 2.73$), the lowest in 30% SW ($K = 1.76$); values refer to a crab with wet weight of 10 g, at 18° C.

3. The calculated net diffusional (osmotic) water influx is adequate to account for the urine production of *Carcinus* in 50–70% SW, but does not account for urine production in SW, and only inadequately for the urine produced in 30–40% SW, and it seems necessary to postulate some isotonic transport of water.

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THE EFFECTS OF ACUTE GAMMA IRRADIATION ON THE BRINE SHRIMP, *ARTEMIA*. I. LIFE SPANS AND MALE REPRODUCTIVE PERFORMANCE[1, 2, 3]

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The variety of metazoans which have been successfully exploited for genetic studies is quite limited, and the majority of such animals are either insects or vertebrates. The range of metazoans used in assaying the effects of radiation on total reproductive behavior has not been much greater. A broadening of the foundation upon which our knowledge of such effects is based necessitates using organisms which are less well known than the standard laboratory species.

Initial reports of Bowen (1962, 1963a, 1963b, 1964), Grosch (1962), Grosch and Erdman (1955) indicated that *Artemia* is well suited as a laboratory animal for studies of a genetic or radiobiological nature. The primary goal of the present study, the first of a series, was to estimate the overall effects of acute gamma radiation on the fitness components of amphigonic *Artemia*. Refined measurements of genetic damage have had to be postponed until after such an analysis.

The experimental design, and effects on life span and male productivity are reported in this paper. Effects on female productivity shall be the concern of a subsequent paper. This is the first and only report concerning the effects of electromagnetic radiation on male reproductive performance in *Artemia*.

MATERIALS AND METHODS

Freshly matured brine shrimp were obtained from two stock #3 culture jars. This stock was derived from commercially obtained Californian cysts, and has been maintained in five-gallon battery jars since 1957 at the Marine Biological Laboratory, Woods Hole. The jars are kept at room temperatures and allowed to evaporate each winter. The cultures are re-established each spring by adding distilled water to the resultant brine and precipitated salts. The spring culture is thus derived from overwintering cysts and a few adults which may still be alive in the brine.

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The culture medium consisted of filtered sea water to which was added 50 g of sodium chloride (Fisher Scientific Co.) per liter (Bowen, 1962). Later, after the surviving animals were transported to Raleigh, North Carolina, "Instant Ocean" (Aquarium Systems, Inc., Wickliffe, Ohio) was substituted for the sea water.

Holding jars were set up eight days prior to irradiation, and young adults were segregated according to sex for the next two days. All adults thus obtained were then held for an additional five days. Eight treatment groups of five males each were assigned by use of a random numbers table to provide irradiated males to be mated to untreated females. This procedure was repeated for females, to provide irradiated females to be mated to untreated males. Separate randomizations then assigned an untreated mate to each experimental animal. Pint-size glass jars, each containing 10 fl. oz. of medium, were used for pair-matings.

For exposure, each treatment group was placed in a small plastic petri dish during exposure and given the appropriate dose of gamma radiation from the M. B. L. cesium-137 unit (Table I). There is no significant heat problem with this source, and room temperatures were thermostatically maintained in the middle seventies (°F)

TABLE I

Male and female adult life spans in days, with two-tailed t test comparisons of treated versus *control animals (maximum range of error in exposure time: ±0.01 min.)*

Level	Dose (kR)	Exposure time (min.)	$\bar{X}$ ± S.E.	t	X ± S.E.	t
1	0	0	52.9 ± 8.5	—	45.6 ± 6.2	—
2	0.1	0.20	49.0 ± 8.7	0.32	43.6 ± 5.5 30.4 ± 5.9[a]	—
3	1	1.00	55.8 ± 4.2	0.31	40.9 ± 8.0	0.47
4	2	2.00	55.7 ± 5.5	0.28	28.7 ± 4.6	2.20
5	5	2.00	48.2 ± 8.0	0.40	40.4 ± 5.8	0.61
6	10	2.05	45.8 ± 5.7	0.70	26.7 ± 5.8	2.24
7	50	10.27	15.9 ± 1.2	4.30**	9.9 ± 0.5	5.79***
8	100	20.54	5.9 ± 0.5	5.53***	4.6 ± 0.6	6.63***

[a] Replication 2 differs significantly from replication 1; t = 2.30.

** Significant at 0.01; ***Significant at 0.001.

All irradiation was completed in a period of three hours, and the animals were then placed in their assigned pair-mating jars. Each jar was examined daily throughout the entire life span of the treated animal. In the treated-male series, females were held for several days after their mate's death, so that any brood which was "in utero" at his death would also be scored. The dead mate of a treated animal was replaced by an adult which was comparable in age to the dead animal, but had not yet reached senility. Replacement females were unmated at the time of use. Scoring criteria consisted of debility or death of the adult animals, and the presence of either cysts or nauplii in the jar.

Female specimens of *Artemia* customarily produce a brood every three and one-half to four days. Oviparous broods (cysts) were filtered out, dried and stored on filter paper. These cysts were later counted and scored for hatchability after hydration in small stender dishes of plain Instant Ocean. Most cysts hatch within 24–48 hours after hydration, but all hatchabilities were scored daily for seven days. Viviparous broods of nauplii were counted immediately.

Broods I and IV were set up in quart jars (each containing 24 fl. oz. of brine), and raised to adulthood. These broods were then scored for morphological abnormalities, sex ratio, and the per cent survival.

Each jar was fed daily with a dilute suspension of Fleishmann's baking yeast and evaporated water was replaced by distilled water. All culture jars received constant overhead fluorescent lighting.

Manipulation of immature and adult animals was greatly facilitated by use of a commercially obtained glass meat baster, used as a giant medicine dropper.

Eleven days later, a second replicate was set up in the same manner as just described, again utilizing five animals of each sex at each of the eight dose levels.

The enormous number of cysts produced by the controls in the treated male experiments precluded a full hatchability analysis for this level, and only a portion of their encysted broods were so analyzed. Except for this parameter, full life history and reproductive data were obtained for each test animal.

RESULTS

Life span

Means and variances were first established within each replicate, and compared by two-tailed t and F tests. The mean values of two replicates were significantly different for female life span at the 100 R level, and this level was excluded from further analysis. The differences of the other means between replicates within treatment levels were not significant. Some of the differences between variances were significant. Average mean values and pooled variances (Steel and Torrie, 1960) were obtained independently for the members of each sex within each treatment level. Comparisons between treatment levels were made for each sex, using a two-tailed t test (Table I).

Exposures of 50 and 100 kR significantly reduced male and female life spans. Reductions in female life span approach significance after doses of 2 or 10 kR, and are significant at the 0.10 level. The male and female dose-effect curves are linear on a semi log plot for the doses of 10, 50 and 100 kR, although the slopes of these lines are quite different, and demonstrate the consistently greater longevity of the homogametic male.

Male reproductive performance

Number of broods. Comparisons of the average number of fertile broods per male are shown in Table II. While 1 kR did not significantly reduce the number of broods per male, 2 kR caused an evident decrease. The differences between the results from 2, 5, and 10 kR are not significant. After 50 and 100 kR, no broods were produced. For purposes of the present study, fertility was defined as the production of nauplii from either a viviparous brood, or from cysts. The voiding of cysts does not in itself indicate male fertility, since many females are capable of depositing unfertilized cysts (which always fail to hatch). Therefore, only fertile broods were considered in this study. Complete genetic dominant lethality is not distinguishable from sperm inactivation or failure of copulation.

Reproductive span. The average number of days during which males were able to sire offspring was calculated per treatment level (Table II). Examination of the records of individual males indicated three distinct categories, a short reproductive span of 11.9 days, a transitional situation, and a long reproductive span of 50.4 days. The short-span animals were found at the 5 and 10 kR irradiation levels. Five of the eight fertile males given 2 kR also belonged to this group ($\bar{X} = 11.8 \pm 0.9$). The remaining three males had a long reproductive span which raised the group's average to the tabulated 28.4 days. However, two of these three males receiving 2 kR, were temporarily sterile from day 13 to day 29 or 36, after which fertility was regained. At doses below 2 kR, no sterile periods occurred and all males exhibited long reproductive spans.

TABLE II

Average number of fertile broods and reproductive span per irradiated Artemia *male, with t test comparisons (one-tailed) of treated* versus *control animals*

Level	Dose (kR)	Number of fertile broods		Reproductive span (days)		Over-all reproductive span
		$\bar{X}$ ± S.E.	t	$\bar{X}$ ± S.E.	t	X ± S.E.
1	0	10.4 ± 1.9	—	53.7 ± 8.1	—	50.4 ± 3.3
2	0.1	8.2 ± 1.4	0.928	44.1 ± 6.4	0.932	
3	1	9.8 ± 1.1	0.276	52.0 ± 4.1	0.187	
4	2	4.0 ± 1.4	2.700*	28.4 ± 8.7	2.126*	
5	5	1.7 ± 0.5	4.416**	11.1 ± 1.4	5.195**	11.9 ± 0.7
6	10	2.2 ± 0.6	4.121**	12.6 ± 1.7	4.951**	

* Significant at 0.05.
** Significant at 0.01.

On day 46 of replicate 2, the three surviving males of the 5 kR exposure and four surviving males which received 10 kR were scored as aspermic by gross observation. Males at lower levels had cellular material present in their seminal vesicles. However, two of the three males which received 2 kR failed to produce additional nauplii.

Unlike the female response to radiation (data to be presented in paper II of this series) fertility in male brine shrimp appears to be unrelated to life span. A short reproductive span was not correlated with life span.

Cyst hatchability. Hatchability tests were conducted for all oviparous broods. However, analysis was restricted to those encysted broods which (1) were not completely infertile, (2) contained at least 25 cysts (*i.e.*, the majority), and (3) were not derived from partially viviparous broods. The percentage of hatched cysts was calculated for each brood. The arcsin $\sqrt{\%}$ transformation was then obtained for each value (Steel and Torrie, 1960), since it is not legitimate to use the untransformed values for the standard error. Untransformed treatment means and t test comparisons are given in Table III. The number of encysted broods per level which could be utilized varied considerably, and only part of the cysts produced by

TABLE III

Artemia *hatchability per fertile encysted brood, with t test comparisons of treated* versus *control animals. The males received an acute gamma ray dose*

Level	Dose (kR)	% hatch		No. of broods scored
		X	t	
1	0	24.3		12
2	0.1	27.4	0.472	27
3	1	15.2	1.583	49
4	2	21.4	0.445	13
5	5	13.7	1.366	3
6	10	6.5	3.200**	13

** Significant at 0.01.

unirradiated males were scored. These control data are in close agreement with the control values obtained in the irradiated-female experiment (Squire, in preparation).

The available sample size at 5 kR is too small to warrant conclusions. Hatchabilities were significantly reduced after 10 kR. This decrease may be ascribed to: (1) failure of some eggs to be fertilized, or, (2) genetic lethals which act prior to the nauplius stage. Thus we consider cyst hatchability as a measure of "dominant" lethal events. We may then calculate the percentage of dominant lethal events as:

$$\%\ \text{DLE} = \frac{\%\ \text{control hatch} - \%\ \text{treated hatch}}{\%\ \text{control hatch}} \times 100$$

This calculation gives an approximate value of 73% for 10 kR.

Survival to adulthood, sex ratio, and X_1 productivity. Broods I and IV were scored for survival to adulthood and sex ratio. The observed values were highly

TABLE IV

Survival to adulthood, sex ratios and morphological abnormalities of X_1 animals fathered by gamma irradiated male brine shrimp

Level	Dose (kR)	% survival to adulthood		No. of broods produced	Abnormalities		Sex ratio % males
		X ± S.E.	t		fraction	%	
1	0	62.5 ± 4.5		14	1/474	0.2	54.6
2	0.1	58.3 ± 5.3	0.579	12	6/584	1.0	49.5
3	1	69.9 ± 2.1	(1.370)	14	3/564	0.5	52.3
4	2	65.7 ± 6.9	(0.378)	8	0/322	0	52.2
5	5	56.2 ± 2.9	1.094	7	6/244	2.5	53.3
6	10	28.2 ± 2.2	4.040**	4	2/50	4.0	60.0
X^2 comparison							5.25NS

** Significant 0.01.

heterogeneous and showed no obvious correlation with (1) brood size, (2) deposition as a viviparous *vs.* oviparous brood, or (3) the length of the post-irradiation period. All viviparous broods were maintained under the same conditions at the M. B. L., Woods Hole. All oviparous broods were maintained under comparable conditions at Raleigh.

Since the results did not differ greatly between broods I and IV, the data were pooled for each treatment level and analyzed on a per-brood basis using a one-tailed t test. After pooling, these values demonstrate a highly significant reduction at 10 kR, but not at lower doses. Sex ratios were not significantly altered at any dose level (Table IV).

In addition, some data were obtained for the reproduction of animals from brood I. The surviving offspring of 10 kR males died early and were usually sterile. Some of these animals were full-sib mated while others were mated to normal animals. Some were mated to normal animals after their sib died. A total of three fertile broods was produced. At 5 kR, a total of 39 full-sib matings resulted in no viviparous broods and a low frequency of oviparous broods. Adult mortality was still very high. At 2 kR, oviparous broods were more frequent, viviparous broods rare, and adult mortality high. At lower dose levels, oviparous broods were common, while viviparous broods remained relatively rare. Adult mortality was not excessive. Labor and space requirements made it impossible to conduct full life history studies for these X_1 animals and most cyst hatchabilities were not analyzed. Consequently, observations had best remain on a qualitative basis.

The number of morphological abnormalities in broods I and IV are summarized in Table IV. Four of the level 5 animals were females. One of the level 2 animals was too immature to sex. The other 13 animals were males. The abnormal females consisted of: (1) one with a pair of male-like claspers and an ovisac full of yolky-appearing material which did not resemble normal eggs, (2) one with a stubby tail and paired ovisacs, (3) one with an undeveloped tubular ovisac and an arched tail, and (4) one with a bulbous distal section of the ovisac. This section is normally thin and tubular, with its long axis parallel to that of the trunk. The axis of this ovisac was ventrally perpendicular to that of the trunk. Abnormalities in the males included eyes which were mosaic for normal pigmentation, shortened or absent claspers, stubby tails, missing caudal furcae, abnormal seminal vesicles, bent tails and apparently fused phyllopodia. Most of these abnormalities were bilaterally asymmetrical.

Discussion

Life span

These results suggest a threshold for obvious life span effects in the 10 kR region, but data are not available to check additional points in the 10–100 kR range against the linear semi-log plot. Ballardin and Metalli (1966) concluded that the life span values for several unspecified strains of *Artemia* may be fitted by an exponential function of the dose after acute exposure of 10, 20, 30, 40, or 50 kR rad. While the curves for diploid and tetraploid females, both bisexual and parthenogenetic, and for tetraploid males show no appreciable shoulder, that for the bisexual diploid males shows a definite shoulder; and these males are more resistant than the other animals. Their abstract does not specify the actual life span values. Grosch and Erdman (1955) also reported males to be more resistant than females, and the data indicated an obvious effect in the 10 kR region. Culture techniques were still sub-optimal in 1955, however, resulting in decreased life span values for both control and treated animals.

The assumption that adult brine shrimp lack tissues with proliferative cells is not valid. Lochhead and Lochhead (1941) found frequent mitotic figures in the hemopoietic organs, and M. Lochhead (personal communication) has observed occasional mitotic figures in the gut as well. To what extent these somatic divisions may be necessary for adult survival is not known. According to Lochhead and Lochhead (1941), hemopoietic mitoses increase in frequency after physical injury. The blood cells thus produced are phagocytic, and perhaps also perform some nutritive function in oogenesis. Conceivably these cells function in tissue replacement necessary for survival, and consequently *Artemia* is more sensitive to radiation than the adult form of the holometabolous insects.

The extent to which the nonlocalized centromere of *Artemia* chromosomes (Stefani, 1963) confers somatic cell resistance to radiation damage is not yet known. The retention of radiation-induced fragments over many cell generations has been repeatedly demonstrated in animals which have holokinetic chromosomes, although mosaic fragment loss may also occur (Barry, Guthrie and Dollinger, 1967; Hughes-Schrader and Ris, 1941; Hughes-Schrader and Schrader, 1961; Nelson-Rees, 1962; Ris, 1942; Tazima, 1964; Tazima and Onimaru, 1969). As Stefani (1963) pointed out, such a radiation study should be conducted in *Artemia*. Grosch and Erdman (1955) noted that a source of radio-resistance may be the somatic polyploidy present in some tissues.

Radiation-induced shortening of life span must be viewed as a summation of innumerable deleterious effects on the animal's physiological processes and cannot be ascribed to genetic damage alone. In this species, the female is the heterogametic sex; and it is the heterogametic sex which is usually less resistant to environmental stress and often short-lived.

Male reproductive performance

Reproductive span. Since there are no reports concerning the effects of electromagnetic radiation on male reproductive performance in brine shrimp, we are forced to compare *Artemia* to such distant relations as the insects and mammals. Such comparisons must consider the anatomical, physiological, and genetic differences which separate such diverse organisms. In *Artemia*, spermatogenesis is continuous and its stages may occur in waves of partially synchronized activity. All stages are present in the mature male (Fautrez-Firlefyn, 1951). The sperm are aflagellate and may be amoeboid (Fautrez-Firlefyn and Fautrez, 1955). Stefani (1963) found the chromosomes to be polycentric in the species of *Artemia* which he studied. Finally, *Artemia* is aquatic rather than terrestrial.

A dose of 2 kR is enough to destroy or incapacitate the spermatocytes of most organisms. While this dose may allow some sperm cells to be formed, most of these spermatozoa carry dominant genetic defects which cause zygotic death early in embryonic development. In the present study which requires decisions based upon interrupted cyst deposit and failure in larval emergence as well as the absence of naupliar production, sterility of the male must be assessed in terms of any cessation of visible offspring from his mate. Nevertheless 2 kR as the sterilizing dose for spermatocytes is consistent with results for some insects, for example *Bombyx* (Sado, 1961; Tazima, 1961, 1964). Diptera are more sensitive, with 1.5 kR the upper limit (Riemann, 1967). Mice may be in another class entirely since less than 700 R suffices (Davies and Evans, 1966; Mandl, 1964).

Spermatogonial response to radiation varies considerably according to the organism and the type of spermatogonial cell involved. In the mouse, 300 R destroys "type B," intermediate, and most "type A" spermatogonia. One resistant component of the "type A" population survives, and eventually multiplies to regenerate the germinal epithelium (Oakberg, 1965). A similar result occurs in *Bombyx* after 2 kR (Sado, 1961). Spermatogonial LD-50's include about 1 kR for *Bombyx* and 23 R for mice.

We conclude that the short fertility period observed in specimens of *Artemia* after 5 and 10 kR represents radio resistant cells which were sperm and perhaps spermatids at the time of treatment. Since the reproductive pattern for most 2 kR males is the same as that for higher doses, the same conclusion seems justified for this level as well. The presence of cellular material in some seminal vesicles of 2 kR males 46 days after treatment, the temporary sterility of two 2 kR males, and the lack of sterility of one 2 kR male all suggest spermatogonial regeneration at this treatment level. The failure of some of these males to regain fertility may be due either to high genetic dominant lethality or to low sperm concentration. The latter could result from incomplete testis replenishment by spermatogonia (Gillette, Hopwood, Carlson and Gassner, 1964). This replenishment and subsequent spermatogenesis is believed to have obscured alteration of the reproductive pattern in the 2 kR male which lacked a sterile period. Replenishment probably had an even greater influence at 1 kR, where no sterile period was observed; also some of the primary spermatocytes may have survived destruction.

Since all surviving males at 5 kR and 10 kR levels were observed to be aspermic on the 46th day after treatment, either all "type A" spermatogonia were destroyed or testis replenishment was insignificant at these doses. The actual duration of the initial fertility period, and hence the subsequent sterility period, is a function of the rate of sperm utilization by the irradiated male. In turn this may depend upon the availability of females and their reproductive cycle. In our experiment, each male was isolated with a single female. If each male had been placed with a fresh receptive female daily, the period of fertility might have been shortened.

Furthermore, the present results were obtained with freshly matured males. Conceivably, slightly older males would contain a greater number of "type A" spermatogonia. If this is the case, more rapid testicular replenishment might result in those males.

Cyst hatchability. In general, spermatids are believed to be more sensitive than mature spermatozoa to the effects of irradiation (Mandl, 1964). The doses required to induce 99% dominant lethal events in the mature sperm of many insects with monokinetic chromosomes are in the range of 3 kR to 11 kR (LaChance, 1967). With holokinetic chromosomes, doses have ranged from 4 kR to 100 kR (LaChance, Schmidt and Bushland, 1967; North and Holt, 1968a, 1968b). Despite considerable differences between insects and shrimp, the estimate of 73% dominant lethality at 10 kR seems reasonable. Indeed the dose required for *Artemia* seems to be rather low for a holokinetic species, but well within the range of other reported values. Possibly treated spermatids as well as sperm contributed to this value. Such a heterogeneity would be expected to lower the observed resistance values.

The low hatchability value for the 1 kR males is probably fortuitous. Upon examination of individual male records, it was found that the average hatchability is no different for the first 15 days than it is for the remainder of the reproductive span. (The untransformed average values were 16.2% and 14.9%, respectively.) This observation rules out the possibility that the lowered value was due to irradiation effects on meiocytes and earlier stages which were excluded from analysis of the higher levels. If this had been the case, then the average values for hatchability would be expected to be lower in the second period which reflects pre-spermatid stages of treatment.

In *Artemia*, the date of brood deposition cannot be accurately correlated with the cytological stage present at the time of irradiation for several reasons: (1) The post-irradiation interval also reflects the reproductive cycle of the female, since only metaphase I eggs are sampled. (2) The normal duration of each spermatogenic stage, the effects of irradiation on the duration of each stage, and the effects of the lower doses of radiation on the survival of these stages are unknown for *Artemia*. (3) The male reproductive system consists of a pair of genital tracts without a common penis. Thus a single fertilization reflects a contribution by one of these tracts, but two successive broods may represent the same tract twice or each tract once. (4) Each testis is a long tubular organ with spermatogonia present along its periphery. A given ejaculate may therefore contain sperm which are heterogeneous in terms of the developmental stage irradiated. Such heterogeneity might be increased by delayed utilization of sperm from males not subjected to sperm exhaustion by multiple mating.

It has been tacitly assumed that the various secretory cells of the anterior testis have not been damaged. While such cells have been relatively radio-resistant in other animals, no data exist for *Artemia*. A hypothetical threshold at 2 kR for these cells would not be inconsistent with the data, if the observed effect were delayed.

Survival to adulthood, sex ratio, and X_1 productivity. By combining data on survival to adulthood with the information gained for the mortality and reproductive behavior of X_1 specimens of *Artemia*, we may draw some general conclusions. After an adult male has received 10 kR, most of his potential offspring are eliminated prior to the naupliar stage. Those which become nauplii show a pronounced mortality rate prior to maturity. Those which reach maturity continue to exhibit a high mortality rate and are almost uniformly sterile. A similar, but less pronounced pattern is indicated for the 5 kR group. Morphological abnormalities are relatively common in both of these groups. The 2 kR level is notable for its lack of demonstrable effect on hatchability, survival to adulthood, or morphological abnormalities. Effects were still observed in the adult X_1 of 2 kR males in terms of reproductive behavior and mortality.

The mosaic nature of morphological abnormalities has often been ascribed to alteration of one-half of a DNA helix following sperm irradiation in other organisms. We cannot restrict ourselves to this interpretation in *Artemia*. The holokinetic nature of the *Artemia* chromosome is expected to reduce fragment loss following irradiation. However, small fragments may still disappear from some cell lines, and thus produce a mosaic individual. Recently, Tazima and Onimaru (1969) reached the same conclusion in *Bombyx* where an exponential increase in mosaic frequency was observed in a dose-effect study of irradiated sperm. An earlier report of mosaic fragment loss is that of Nelson-Rees (1962). Incomplete penetrance of induced mutations might also be reflected in asymmetry.

The data for the 10 kR level suggest that the X_1 is much less fertile than are the irradiated males themselves, a sterility pattern typical of species with holokinetic chromosomes. Investigated cases have involved exposure of post-meiotic stages, often mature sperm, to sterilizing doses of radiation. Since the effect of single-break aberrations is minimized by the holokinetic nature of the chromosome, the sterility pattern is believed to reflect multi-break phenomena, and in particular, translocations (Bauer, 1967; Nordenskiold, 1963; North and Holt, 1968a, 1968b; Walker and Quintana, 1968). Irregular fragment behavior and inversion heterozygosity may be additional factors, and Nordenskiold (1963) found in the plant *Luzula* that if a holokinetic chromosome is fragmented into more than two pieces, subsequent recombination with an unfragmented homologue will yield a secondary fragmentation of that homologue. Meiotic segregation may then result in chromosomal imbalance and sterility.

The failure to detect a significant alteration in sex ratio may be due either to a reduced sample size at 10 kR, or to an insufficiently large dose of radiation. Grosch (1962) observed that the sex ratio of *Artemia* tended to favor males when the parents were of irradiated ancestry. This tendency was especially pronounced in populations which were on the verge of extinction. Grosch correlated this effect with female heterogamety.

We would like to thank Mrs. Sally Barnes and Mrs. Ann G. Spencer for the technical assistance provided during this experiment.

Summary and Conclusions

Life span of both sexes

Freshly matured male and female specimens of Californian *Artemia* (stock #3) were irradiated with acute doses of 0, 0.1, 1, 2, 5, 10, 50, or 100 kR gamma radiation and pair-mated to untreated animals. Significant reductions in life span resulted after 50 or 100 kR to either sex. Reductions in female life span approached significance after 10 kR. This moderate radiosensitivity can be explained if cell proliferation in some adult tissues improves the probability of individual survival.

Male reproductive performance

Treated males were completely sterile after doses of 50–100 kR. Males which received doses of 5 or 10 kR had an initial fertility period lasting 12 days, followed by permanent sterility. After a dose of 2 kR, 7 out of a sample of 8 males demonstrated the same initial period of fertility, followed by a period of sterility. Two of the 7 subsequently recovered their fertility. No sterile period occurred after 0.1 or 1 kR. These results are interpreted as indicating spermatogonial stem-cell replenishment of the testicular epithelium in some males after 2 kR and in all males irradiated with 1 kR. It is further hypothesized that all spermatocytes were in some way incapacitated or destroyed following doses above 2 kR.

Hatchability data failed to detect induced dominant lethality in sperm (and perhaps spermatids) after 2 kR. Induced damage resulted in 73% dominant lethal events at 10 kR. No effect on other stages was observed at less than 2 kR.

In the X_1 generation, a dose of 10 kR reduced survival to adulthood, fertility, and adult life span. Similar, but less pronounced effects were obtained after 2 and 5 kR. These data indicate induced genetic damage. No significant alterations were observed in sex ratios.

X_1 sterility appeared to be greater than that observed for irradiated sperm. This point is being reinvestigated, and would be compatible with the reported holokinetic nature of the *Artemia* chromosome.

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THE EFFECTS OF ACUTE GAMMA IRRADIATION ON THE BRINE SHRIMP, *ARTEMIA*. II. FEMALE REPRODUCTIVE PERFORMANCE[1, 2, 3]

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The effects of acute gamma irradiation on adult life span and male reproductive performance were reported in the first paper of this series (Squire and Grosch, 1970), along with the Materials and Methods, and the general rationale behind the experiments. The present paper records the effects on female reproductive performance.

The earlier reports of Grosch and Erdman (1955) and Grosch and Sullivan (1955) described some of the criteria of x-ray damage to female reproductive performance. However, these data were dependent upon culture techniques which have since been modified. In addition, these reports did not provide an analysis according to the gametogenic stage irradiated. The report of Grosch (1962) was restricted to the analysis of populations of irradiated ancestry, and did not concern the treated generation itself.

Cervini and Giavelli (1965), Giavelli (1966), Giavelli and Cervini (1966), and Metalli and Ballardin (1962) restricted their studies to fecundity and fertility analyses of viviparous broods after oocyte treatment of various parthenogenetic species of *Artemia*.

RESULTS

Fecundity

The statistical procedures were the same as those described for the life span studies (Squire and Grosch, 1970). Fecundity was defined as the sum total of viviparous (nauplii) and oviparous (cysts) gametes produced by a given female throughout her life. All treatments significantly reduced fecundity (Table I). A dose of 5 kR resulted in greatly reduced fecundity ($\bar{X} = 43$), while doses of 10 50, and 100 kR resulted in total infecundity. Since the mean life span of the 5 kR

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TABLE I

Average fecundity and number of broods per irradiated female, with one-tailed t test comparisons of treated versus *control animals*

Level	Dose (kR)	Fecundity/female		No. of broods/female	
		$\bar{X} \mp$ S.E.	t	$\bar{X} \mp$ S.E.	t
1	0	1695 ∓ 329	—	10.1 ∓ 1.8	—
3	1	919 ∓ 216	1.974*	7.0 ∓ 1.3	1.569
4	2	289 ∓ 134	3.961**	4.2 ∓ 1.1	3.123**
5	5	43 ∓ 17	5.021**	1.1 ∓ 0.3	5.800***

* Significant at 0.05.
** Significant at 0.01.
*** Significant at 0.001.

females was virtually identical to that of the controls, this effect on fecundity is independent of life span.

The mean differences between replicates within treatment levels were not significant for any dose at 0.05. The F test for variances between replicates within treatment levels was significant for level 5 (5 kR). This difference was due to a single female which produced 1255 gametes, when the group average excluding her contribution was 66. She was 17 standard deviations from the mean, and none of her cysts hatched after the first brood. Accordingly the data from this female were not used in the fecundity analysis. Adjusted means, variances and degrees of freedom were used for comparisons involving this treatment level.

Fertility

A similar analysis was conducted for fertility (Table II), which was defined as the sum total of the nauplii produced as viviparous broods plus those which hatched from oviparous cysts. The difference in variances (but not means) was once again significant for 5 kR. The separate means were 4.6 and 0.2. Appro-

TABLE II

Average fertility per female and per cent hatchability per brood, with one-tailed t test comparisons of treated versus *control animals*

Level	Dose (kR)	Fertility/female		% hatchability/brood	
		$\bar{X} \mp$ S.E.	t	$\bar{X}$	t
1	0	1318 ∓ 358	—	18.5	—
3	1	718 ∓ 201	1.46 (0.10)	21.0	(0.503)
4	2	64 ∓ 30	3.49**	12.1	1.824*
5	5	2.4 ∓ 1.7	3.68**	2.2	7.412*

* Significant at 0.05.
** Significant at 0.01.

priate pooled values were nevertheless obtained for all treatment levels and comparisons made (Table II). The lack of significance at 1 kR may be ascribed to the presence of a few females which produced large numbers of cysts with low hatchability. This comparison is significant at the 0.10 level.

As may be seen in Tables I–II, the average fecundity and fertility per female decreases with increasing dose. The same trend is found when the per female values are computed for average brood size, largest brood size per treatment level, the average of the largest single brood for each of the fecund females, or the average largest brood of the three best females (Table III).

Cyst hatchability

Hatchability tests for all oviparous broods were conducted and analyzed in the same manner as previously described for males. Untransformed treatment means and t test comparisons are given in Table II. While 1 kR did not decrease hatchability, 2 kR and 5 kR did so. The differences of 1 kR *vs.* 2 kR, and 2 kR *vs.* 5 kR are also significant.

TABLE III

Evidence for a reduction in the number of competent oogonia as a result of gamma irradiation of adult female brine shrimp

Level	Gamma Ray Dose (kR)	Avg. size of brood		Largest single brood value in treatment level		Avg. of the largest single brood produced by each female		Avg. fecundity of the largest single brood produced by each of the 3 most productive females
		Fec.	Fert.	Fec.	Fert.	Fec.	Fert.	
1	0	168	134	485	485	268	240	423
3	1	130	102	374	374	191	179	310
4	2	69	15	217	99	97	26	177
5*	5	57	1	238	18	71	3	133

* The values for level 5 include the exceptionally fecund female described in the text.

Number of broods

The average number of broods per female was calculated in the same manner as the fecundity data. The reduction in brood number was highly significant at doses of 2 kR and more (Table I). The average interval between broods was calculated per female (Table IV) and found to increase markedly at 2 kR and 5 kR. The average percentage of gametes produced as cysts per female also rises at these levels (Table IV).

Survival to adulthood and sex ratio

The percentage of the nauplii (viviparous or hatched from cysts) to reach maturity was also calculated for broods I and IV. Survival to adulthood measures late dominant lethal events, since, as measured here, it excluded all pre-

TABLE IV

Average brood intervals, percentage of oviparous gametes, and the fraction of encysted broods which failed to hatch after adult females were irradiated

Level	Gamma Ray Dose (kR)	Avg brood interval (days)	Avg % of gametes deposited as cysts/female	Fraction of encysted broods which failed to hatch
1	0	3.74	41.3	1/32
3	1	3.53	38.5	3/21
4	2	6.89	86.4	1/23
5	5	9.08	85.7	14/17*

* Nine-tenths of these broods were produced by one exceptional female; see text.

naupliar deaths from analysis. Analysis of brood I was restricted to those broods which were deposited between the fourth and sixth days after treatment, and thus were derived from irradiated oocytes or oocyte-nurse cell complexes. If the time sequence published for the diploid parthenogenetic species from Sète (Cervine and Giavelli, 1965) is applicable to this species, then most of these oocytes were in prophase I at time of treatment, and some may have reached metaphase I. None of these gametes were post metaphase I at time of treatment, since in order to be fertilized the eggs must be in the oviducts when copulation occurs; and such eggs are in metaphase I at that time.

Brood I was analyzed as 2 × 4 contingency tables (Steel and Torrie, 1960) for survival to adulthood and sex ratio. Survival to adulthood was significantly reduced with increasing dose ($P < 0.005$). There was no significant change in sex ratio (Table V).

Brood IV animals were derived from treated oogonia. The survival and sex ratio data are summarized in Table V. None of these values are significant, although the survival value for level 4 might have reached significance with larger

TABLE V

The survival to adulthood and sex ratio of offspring from ten irradiated females per gamma ray dose level shown

Brood #	Level	Dose (kR)	No. of nauplii	No. of survivors	% survival	Sex ratio % males
I[a]	1	0	157	129	82.2	50.4
	3	1	53	36	67.9	52.8
	4	2	50	21	42.0	52.4
	5	5	18	6	33.0	33.3
X^2					40.25**	0.95NS
IV	1	0	1147	582	50.7	48.7
	3	1	581	252	43.4	49.6
	4	2	55	20	36.4	60.0
	5	5			—	—
X^2					1.04NS	0.18NS

[a] Brood I data selected for days 4–6 only; see text.

** Significant at 0.01.

samples. Survival was also analyzed by using t test comparisons of percentage data following the arcsin $\sqrt{\%}$ transformation, as well as with the contingency table shown.

Additional observations

Gross observation of females of replicate 1 on the 22nd day after treatment revealed full ovisacs in all cases after 5 and 10 kR. Nevertheless, two of the five females given 5 kR and all of the three surviving 10 kR failed to produce recoverable nauplii or cysts subsequent to this observation. No excretory gland abnormalities were noted.

Cysts from irradiated females were often orange and translucent, while normal cysts are opaque and brown. Cysts deposited after 5 kR exposures were particularly abnormal in this respect, and were also characterized by frequent brittleness (which resulted in cyst breakage when touched with the dissecting needle during hatchability studies) and flattened cysts approaching a disc shape. Since all cysts were treated and stored under identical conditions, the abnormality is attributed to experimental treatment, rather than to attendant conditions of the experiment. Broods in which translucent cysts predominated showed poor hatchability and particles resembling cysts occasionally degenerated completely during a brief storage period on filter paper.

Some pair matings were set up from first-brood individuals. Offspring from the 5 kR series failed to reproduce (two full-sib matings plus three females crossed to normal males). Eight full-sib matings from the 2 kR series produced cysts but no nauplii. The offspring from 1 kR females produced both nauplii and cysts. Mortality was high in the 2 kR and 5 kR offspring following sexual maturity. The cysts produced by these X_1 individuals were not tested for hatchability in most cases, and full life history data were not obtained.

A few morphological abnormalities were scored in the X_1 of treated females. Unfortunately, these animals usually failed to reproduce. The fusion of adjacent appendages to each other, or to genitalia, was the most common trait. Abnormally small eyes or claspers were asymmetrical traits, and abnormal reproductive organs (only one functional ovary), bent tails, missing eyes or claspers were also noted.

DISCUSSION

Factors affecting female reproductive performance

Female fecundity was defined as the sum total of all recoverable gametes produced by a single female throughout her reproductive history. Such a measure is the final result of many interacting factors. Presumably a cohort of oogonia is produced from a smaller number of stem cells. On an average of every three and one-half days, these oogonia then produce a ribbon of cells which in time differentiate into an oocyte and a nurse-cell complex. Nurse cells become polyploid and contribute to vitellogenesis. The blood cells have also been suggested to play a role by transporting materials to the ovarian region. Finally, a differentiated oocyte is produced which enters the median ovisac (uterus) and is quickly fertilized. (For a more comprehensive coverage, see papers by Anteunis, Fautrez-Firlefyn and

Fautrez, 1966a, 1966b; Bowen, 1962; Cassidy, 1965; Fautrez-Firlefyn, 1951; Lochhead, 1950; Lochhead and Lochhead, 1941, 1967.)

In the author's opinion, *Artemia* oogonia comprise a nonexhausting stem-cell population in the adult female; and a single oogonium may contribute to each successive brood. According to this view, the stem-cell population would be comprised of "primary oogonia." Every three and one-half days, each primary oogonium would divide, producing one primary oogonium and one "secondary oogonium." This secondary oogonium would then undergo a series of mitotic divisions and produce an oocyte-nurse cell complex.

Normally, the second cleavage division occurs about five hours after the descent of the eggs into the uterus, and shell formation is initiated at this time in oviparous broods (Fautrez-Firlefyn and Van Dyck, 1961). Unfertilized eggs may also be encysted (Squire, unpublished data). In the case of viviparous broods, the zygotes must differentiate into a swimming nauplius in order to be scored. In the case of oviparous broods, any gamete which passes into the uterus will probably be recovered so long as shell deposition is approximately normal. Cyst hatchability, then, is a measure of dominant lethal events. Factors which may conceivably affect cyst hatchability include (1) failure of fertilization, (2) nutritional inadequacies of the oocyte resulting from damage to the oocyte, to the nutritive cells of the complex, or to general physiological disturbances in the females, (3) improper shell deposition, and (4) genetic lethality. Reabsorption, either of gametes prior to fertilization, or of viviparous zygotes after fertilization, might also be a factor in scoring fertility and fecundity.

Fecundity was defined as the total number of recoverable gametes. Thus, it would include (1) any change in the number of oogonia actually present at the time, (2) induced changes in the inherent capacity of those oogonia which were present to produce functional gametes, (3) any nutritional changes which led to the failure of a differentiating oocyte to be recovered (such as the absence of vitellogenesis or reabsorption of the oocyte), (4) reabsorption of viviparous zygotes and (5) failure to score partially developed viviparous zygotes, which had been expelled, prior to their disintegration.

If the concept of an oogonial stem-cell component is correct, then any change in the general health of a female *Artemia* may temporarily or permanently alter the number of gametes recovered per brood. Such a decrease could occur without altering the actual number of oogonia present in the ovaries. Although we have no information concerning the effects of irradiation on the physiology of *Artemia,* presumably such effects exist, and quite possibly nutrient utilization is altered.

Some information does exist concerning various aspects of brine shrimp nutrition. Lochhead and Lochhead (personal communication) reported that oocytes may fail to differentiate in starved animals. They believe that oogonial number is more strongly influenced by nutrition than by age. Subsequent events in our laboratory would tend to support some of their conclusions. Greatly reduced fecundity and stage-specific patterns of larval mortality appeared to be associated with a lack of algae in the diet.

D'Agostino and Provasoli (1968) demonstrated that the inter-relationship of algal diet, salt concentration, other nutritional requirements, and fertility is not a simple one. Reduced salinity resulted in depressed fertility unless specific nutrients were added to their synthetic media.

Fertility, defined as the sum total of the recovered nauplii from viviparous and oviparous broods, combines the factors included in fecundity and hatchability. It should be pointed out that we still do not know what factors control the mechanism of cyst deposition. The frequency of cyst production is generally felt to increase as a response to stress, but normal females also produce oviparous as well as viviparous broods. Various environmental factors such as diet, temperature and salinity have been implicated at one time or another.

Fecundity of irradiated females

Female fecundity was progressively reduced at doses of from 1 kR to 5 kR. No recoverable gametes were found at 10 kR or higher doses. The data summarized in Tables I–III indicate that the total number of oogonia which successfully contribute to each brood has been progressively reduced with increasing doses. However, no cytological study of the number of oogonia actually present in these females has been made.

The largest single brood per female, the single largest brood per treatment, and the average of the three largest brood values (on a per-female basis) per treatment group all progressively decrease with increasing dose. This leads me to hypothesize that gonial cell lethality occurs at all doses of 1 kR and above, although the other factors discussed above should not be ruled out.

Typically, the first brood produced by a female *Artemia* is relatively small, but subsequent broods are larger. This suggests that the young female may have fewer competent oogonia capable of contributing to each brood, but the number of such oogonia increases rapidly following sexual maturity. Whether this increase is due to recruitment from already existing "dormant" oogonia or to multiplication of stem-cells cannot be determined at the present time. In either case, a differential radio-sensitivity is likely to occur, with actively dividing cells being the most sensitive to damage. Such damage would be expected to give the kind of results reported here.

Although two reports (Grosch and Erdman, 1955; Grosch and Sullivan, 1955) indicated the sterilizing dose for premeiotic and early postmeiotic stages, there have been no reports directly concerned with gonial cell sensitivity in *Artemia.* The silkworm, *Bombyx,* is perhaps the closest biological system with which we can currently make comparisons. This Lepidopteran has 28 pairs of minute and presumably holokinetic chromosomes, as compared to 21 pairs of holokinetic chromosomes in the common Californian race of *Artemia.* Tazima and Kondo (1963, page 246) concluded that the LD-50 is about 1000 and 2000 R for spermatogonia and oogonia, respectively, when exposed to Cs-137 acute gamma irradiation. Ballardin and Metalli (1968) state that an oogonial dose of 1 kR had no effect on the fecundity of the diploid parthenogenetic species from Sète, when measured over a 20-day period. Their abstract gave no data concerning this point.

Cyst production and hatchability

The hatchability data show no detectable dominant lethality at 1 kR, although such events do occur at 2 kR and higher doses. The high frequencies of abnormal cysts recovered from 5 kR females suggest that the shell glands are damaged by this dose, and lower levels may also have been affected. Such abnormal shell deposition could easily alter the resistance of the embryo to normal environmental conditions such as drying, and thus by reflected in hatchability and fertility data. Since cysts from unfertilized females appear normal, it is concluded that genetic dominant lethals which were induced in the oogonia would not affect cyst morphology.

The analysis of hatchability data excluded all encysted broods with zero hatchability. This exclusion does not seriously affect the data as summarized in Table II for the following reasons. Such broods are extremely rare at all levels except 5 kR (Table IV). At this level the average hatchability already approaches zero ($\bar{X} = 2.2\%$). The inclusion of broods with zero hatchability would reduce this value still further, while values for the other levels would remain substantially the same as presented. Excluding such broods is an attempt to separate other dominant lethal events from the failure of fertilization. The latter condition will produce broods with zero hatchability.

Repeated failure of copulation often results in the accumulation of several unexpelled egg clutches within the female. Gamete degeneration becomes evident, with pronounced accumulations of yolky material and darkening of the excretory glands (Lochhead and Lochhead, 1941) which may result in temporary or permanent infecundity (M. Lochhead, personal communication). Some females successfully expell numerous egg clutches in the absence of copulation and thus escape this syndrome. Expelled material is usually in the form of cysts, but undeveloped products lacking cyst walls have been recovered. Expulsion of partially developed embryos has also been observed in females after a fractionated dose of 5 kR (1 kR of gamma radiation per day for five days; Squire, unpublished data). These embryos degenerate rapidly at summer temperatures and are easily overlooked. On the other hand, on several filter papers used to separate encysted broods I failed to find cysts after a storage period and suspect that the objects filtered may have been undeveloped eggs without cyst walls.

The average per cent of gametes deposited per female (Table IV) must be viewed with these factors in mind. Nevertheless the difference in values at 2 kR and 5 kR is outstanding. This difference becomes even more remarkable upon observing that at 5 kR all recoverable broods consisted solely of cysts when premeiotic stages were irradiated, and the same was usually true for the 2 kR series as well. Viviparious broods were frequent after 1 kR and in the controls.

Brood interval and number of broods

The average interval between broods also increased in the 2 kR and 5 kR levels (Table IV). Examination of individual female records suggest this increase was due to the failure of whole broods to be recovered, rather than to a simple lengthening of the interval between broods. This observation cannot be explained simply by complete dominant lethality in the 2 kR series, since most encysted broods showed some hatch; and a number of viviparous broods were scored. Complete dominant lethality could account for the absence of viviparous broods in the 5 kR series, however, since cyst hatchability approached zero for this group. Failure of fertilization may also explain the 5 kR results, but once again the relative infrequency of encysted broods with zero hatchability makes this explanation difficult to accept for the 2 kR series (Table IV). The observation of Grosch and Erdman (1955, page 280) bears repeating: "the neighborhood of 2000 R is critical not only for numbers of broods produced but on the basis of inhibition of viviparity."

In earlier research, an acute dose of 2500 R x-days resulted in a cessation of female gamete production, while 2250 R or less did not prevent continued reproduction by cells which were premeiotic at the time of treatment (Grosch and Sullivan, 1955). Acute x-ray doses of 4080 R or more, usually resulted in females with empty uteri, although exceptional females produced a single brood from cells which were prezygotic at the time of irradiation (Grosch and Erdman, 1955). In the present experiment, full uteri were observed at 5 and 10 kR, although no gametes were recovered from 10 kR females and 5 kR females were highly infertile. The earlier and recent experiments differed in several factors. In 1955 sea water rather than brine was used. This resulted in a shortened life span for irradiated and control animals, and probably imposed an additional stress as well. The reproductive behavior of treated specimens of *Artemia* would thus reflect the combined effects of stress due to irradiation and suboptimal salinity. Squire (unpublished data) found that the fertility of stock #3 animals cultured in plain Instant Ocean was only 14% that of animals maintained in the improved medium, while Grosch (1962) found that the adaptive values of experimental cultures varied with salinity.

In the present experiment, several 5 kR females were characterized by extremely late dates of brood I deposition. A detailed analysis shows that of the 10 females treated, one produced 11 broods, four produced two broods each, two produced one brood each, and two failed to produce any recoverable broods. In all but one case, these broods were probably oogonia at the time of treatment, suggesting that 5 kR resulted in complete dominant lethality of oocytes irradiated prior to metaphase I.

When the life span data of Grosch and Erdman (1955) are superimposed on the present reproductive histories of various treatment levels, the number of broods per female is quite similar in the two experiments. We may therefore conclude that most, if not all, of the discrepancies between the two experiments stem from differences in life span. As they point out, the first brood deposited in their experiment usually represented cells which were postmeiotic at the time of treatment.

X_1 mortality, sex ratio, and reproductive performance

Brood I data for survival to adulthood, adult mortality and reproductive patterns demonstrate the presence of genetic damage in those animals descended from 2 kR and 5 kR females. Genetic damage at a lower dose has been reported for diploid parthenogenetic *Artemia* from Cagliari. A reduction in the fertility of irradiated and X_1 females followed a 1 kR dose of x-rays to prophase oocytes (Metalli and Ballardin, 1962). This reduction was significantly greater in the X_1 than in the treated generation. They obtained similar results with the tetraploid parthenogenetic species from Comacchio, except that the effect was less pronounced, and there was no significant difference between the X_1 and treated generations.

Survival to adulthood of nauplii or hatch derived from irradiated oogonia (brood IV) demonstrated a decrease in average values with increasing dose, but was not significant. Ballardin and Metalli (1968) state that an oogonial dose of 1 kR had no effect on the survival to adulthood of X_1 diploid parthenogenetic *Artemia* from Sète.

As would be expected, the results of various radiation experiments differ according to the polyploid level and the type of meiosis (regular or variously modified) characteristic of the particular *Artemia* species. Additional data from these species will provide a unique opportunity for comparative studies.

The failure of any treatment to seriously affect the sex ratio suggests that the differential segment of the Y-chromosome may not contain many viability loci in the heterogametic female.

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Summary and Conclusions

Treated females were completely infecund after doses of 10–100 kR. Reduced fecundity resulted from doses of 1–5 kR. This is ascribed to oogonial lethality, and it is proposed that the *Artemia* ovary contains a non-exhausted oogonial stem-cell component which contributes to each successive brood. Additional causes of reduced brood size may be nutritional inadequacies and other physiological damage induced by the treatment. Sterility was almost complete after 5 kR.

Cyst hatchability data revealed no detectable dominant lethality after 1 kR, although such effects did occur after 2 and 5 kR. Some of the failure in hatching reflects probable physiological damage to the shell glands, as well as other physiological and genetic components.

The average number of broods was significantly decreased after 2 and 5 kR. Much of this reduction resulted from the absence of entire broods from the recorded data. Viviparity was also inhibited at these doses.

Data for survival to adulthood, adult mortality and reproductive patterns demonstrate that genetic damage was present in those animals descended from oocytes which had been treated with 2 or 5 kR. No definite decrease in survival to adulthood was found in animals descended from treated oogonia after 1 or 2 kR. Sex ratios were not significantly changed with any dose.

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STUDIES ON THE BIOLUMINESCENCE OF THE MARINE OSTRACOD CRUSTACEAN *CYPRIDINA SERRATA*[1]

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At present, at least three species of marine ostracods belonging to the family Cypridinidae are known to be luminous. Among these, the luminescence of only one species has been well-studied. This is *Cypridina hilgendorfii* Müller, 1890, a species found in the coastal waters of Japan. Extensive studies carried out during the past 50 years have yielded detailed knowledge concerning the mechanism of bioluminescence. The organism lives in the sand and comes out to feed at night. When mechanically disturbed, the organism produces a blue luminescence by ejecting luciferin and luciferase into the surrounding sea water from two separate glands. The light-emitting reaction involves the oxidation of luciferin by molecular oxygen, catalyzed by the enzyme luciferase.

The second species is *Cypridina noctiluca* Kajiyama, 1912. In contrast to *C. hilgendorfii*, *C. noctiluca* is a free-swimming pelagic form. It is widely distributed along coastal waters in the western Pacific from southern Japan and Hawaii to Australia and Southeast Asia, and in the Indian Ocean. Haneda (1940) observed *C. noctiluca* at Palau Island. When the beam of an electric light was directed into the water where large numbers of the organisms were swimming, a bright luminous response was obtained. When a plus (+) mark was written on the surface of the water and the light immediately extinguished, a luminous plus (+) mark could be observed in the darkness. Other plankton organisms did not give the same response. Haneda (1953) also studied *C. noctiluca* at Hachijo Island, a subtropical island located approximately 200 kilometers south of Honshu, Japan. The secretory behavior and color of light of *C. noctiluca* were the same as *C. hilgendorfii*. Light resulted (positive luciferin-luciferase reaction) when a hot-water extract (luciferin) and a cold-water extract (luciferase) of the organism were mixed. These extracts also gave reciprocal light-emitting cross-reactions with hot- and cold-water extracts of *C. hilgendorfii*.

The third species is *Vargula harveyi*, recently reported from Jamaica, West Indies, by Seliger and McElroy (1965) and described by Kornicker and King (1965). Measurement of the bioluminescence emission spectrum showed a peak at 478 nm, close to the peak of 465 nm for *C. hilgendorfii*.

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The subject of this paper concerns a fourth species, *Cypridina serrata* Müller, 1906. *C. serrata* was collected during the recent R/V Alpha Helix Biological Expedition to New Guinea. Little is known about the distribution of this organism. The specimens studied by Müller (1906) were obtained from three locations: Paternoster Island (Station 40), approximately 118°E, 7°S in the Flores Sea, Indonesia; North Ubian (Station 99), 120°26′E, 6°7′N, Philippines; and Sulu Archipelago (Station 109), Philippines. The present finding of *C. serrata* at Madang, New Guinea, suggests that the organism is a widely distributed species in the western Pacific. We present herein the first observations of luminescence in this organism.

MATERIALS AND METHODS

Cypridina serrata were collected in a cove in Kranket Island next to Dallman Passage in Madang Harbor, New Guinea. They were also found off Beliau Island and in Binnen Harbor, but not in such large numbers. *C. serrata* were located by shining a flashlight into the water in 2–3 second bursts. This caused elliptical clouds of bright blue luminescence, around 2–15 cm long, to appear in the water, presumably as a result of *C. serrata* organisms ejecting luciferin and luciferase into the sea water.

Collection was carried out by towing two weighted 38-mesh plankton nets (2–3 miles/hour) at a depth of about 1 meter below the surface through waters showing the best flashlight response and emptying them periodically into buckets containing sea water.

Towing distance from shore ranged from 2–15 meters along a shore length of approximately 1 kilometer. At high tide, the depth within this area varied between 2–10 meters. An attempt was made to collect *C. serrata* by lowering fish heads attached to strings in the water, the same method used for collecting *C. hilgendorfii* in Japan, but it was unsuccessful. All collections were made between 8–11 PM. Buckets containing the night's collection were strained first through a coarse wire mesh, then through a 74-mesh plankton net screen. When examined microscopically, the filtered material consisted largely of copepods, nonluminous shrimps, siphonophore fragments and *C. serrata*. Among these organisms, only *C. serrata* was found to luminesce spontaneously. Microscopic counts showed 2000–4000 *C. serrata* in a single night's collection. Each such collection was washed with clean sea water, then resuspended in a small volume of sea water and filtered through Whatman #2 folded filter paper. The filtered residue was then either freeze-dried or used directly in preparing luciferase. In the latter case, the entire batch was ground with a mortar and pestle, washed with about 40 ml of distilled water into a dialysis bag and dialyzed for 60 hours in an ice box against 4 changes of distilled water. The contents of the bag were then centrifuged for 30 minutes at $1500 \times g$ in an Aloe conical centrifuge. The supernatant was decanted, the precipitate washed twice with 10 ml of distilled water and centrifuged. The supernatants were combined and dialyzed for 48 hours in the refrigerator against 4 changes of distilled water. The contents of the bag were freeze-dried, redissolved in 12 ml of 0.07 *M* sodium phosphate buffer, pH 6.8, dialyzed 16 hours against the same buffer, and put on a 45 cm Sephadex G-200 column equilibrated against the buffer. The column was eluted with the buffer and 0.02 ml of each collected fraction (fraction volume, 6.3 ml) was assayed for luciferase activity by mixing with 2.0 ml of 0.2 *M* sodium phosphate buffer, pH 6.8, and injecting into a vial containing 0.05 ml of *C. hilgendorfii* luciferin. Light intensity was measured in arbitrary light units using a photomultiplier photometer. The active fractions were combined, dialyzed 60 hours against many changes of distilled water, and freeze-dried. Further purification of luciferase was not carried out due to limited quantities of the organism.

C. serrata luciferin used for kinetic and oxygen-requirement experiments was prepared by grinding 0.4 g of the freeze-dried material in 15 ml of boiling distilled water in an all-glass homogenizer for 1 minute. The suspension was then quickly cooled in an ice bath while bubbling with 99.99% argon. After centrifuging at 12,000 × *g* at 4° C for 8 minutes, the clear straw-colored solution of luciferin supernatant was stored under argon in an ice-bath until used. Luciferin used in the chromatographic experiments was prepared separately, by extracting 0.25 g of powdered organisms in 15 ml of absolute methanol for 5 hours in an argon atmosphere. After centrifuging at 12,000 × *g* at 4° C for 10 minutes, 3 ml of the clear brown supernatant were evacuated to dryness, redissolved in a few drops of absolute methanol, and spotted directly on the Whatman filter paper.

C. hilgendorfii luciferase was prepared in a highly purified form by the method of Tsuji and Sowinski (1961), and in a partially purified form by the same method as for *C. serrata* luciferase for comparative purposes.

C. hilgendorfii luciferin was purified by the method of Tsuji (1955), except that the initial extraction of luciferin from the defatted *Cypridina* powder was carried out with absolute methanol instead of butanol. Nine ml of the final ice-cold butanol solution of luciferin were evacuated to dryness, redissolved in 30 ml of 0.1 *N* HCl, and used directly. For chromatographic experiments, some of the dry luciferin was redissolved in a few drops of absolute methanol, and used directly.

Chromatography of *C. serrata* and *C. hilgendorfii* luciferins was carried out according to the method of Tsuji (1955), using Whatman No. 3MM paper and a solvent mixture of ethyl acetate, ethyl alcohol, and water (5:2:3 by volume). Chromatography was carried out at 23° C for 1 hour and 15 minutes.

Antibody to *C. hilgendorfii* luciferase was prepared as purified gamma globulin fraction from pooled antisera of rabbits immunized against luciferase as described by Tsuji *et al.* (1969).

Light intensity was measured using a photomultiplier photometer and a chart recorder.

RESULTS

1. *General observations*

Cypridina serrata organisms are shown in Figure 1. Two other forms, *Cypridina inermis* Müller, 1906, and *Melavargula* species, of undetermined luminosity, were also collected in small numbers in the same area but were not studied. The specimens were identified (which included dissection studies) by Dr. Louis S. Kornicker of the Smithsonian Institution. Specimens of all three species are on deposit at the U. S. National Museum. The depository numbers and size of two of the specimens of *C. serrata* (dissected) are as follows: 128152, male, length 1.66 mm, height 0.86 mm; 128153, female, length 1.53 mm, height 0.86 mm.

The bright bluish luminous clouds, produced by *C. serrata* when stimulated with a flashlight (see *Discussion* section), were beautiful and impressive, and probably represent a unique display among luminous organisms. Short 1 second bursts of light produced relatively few clouds and 2 second bursts produced the maximum number of clouds, approximately 50–100 per cubic meter of water. Spontaneously produced luminous clouds were not observed in the water. The response to a single stimulation was strikingly uniform: the luminous clouds burst instantly and decayed within 3–4 seconds after the flashlight was turned off. A given water volume usually responded 3–5 times to the flashlight before becoming refractory to further stimulation; that is, shining light into the water did not elicit any more luminous clouds. The interval between flashlight bursts was 4–5 seconds. A refractory water volume did not respond to further flashlight stimulation, but after remaining in darkness for 20 minutes a few luminous clouds could be produced.

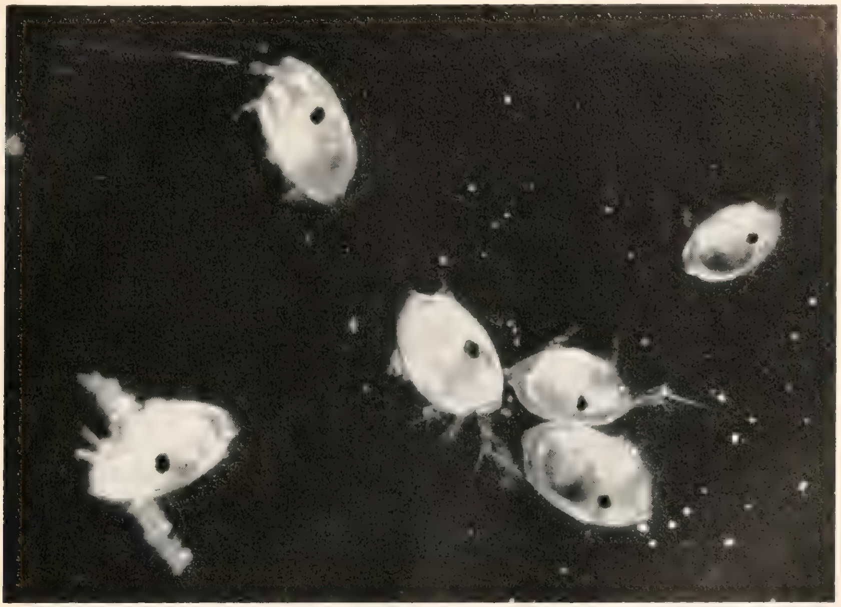

FIGURE 1. *Cypridina serrata,* actual size about 1.6 mm; Madang Harbor, October, 1969.

Many of the luminous clouds were located 1 meter or more below the surface of the water. Often when these clouds were scooped up with a plankton dip net, a bright blue luminous spot was detected on the net and from it a copious luminous secretion soon began to flow down the side of the net. When the organism was touched or gently teased off the net with a finger for identification, the finger became intensely streaked. These large bright blue spots, often continuously luminous, are to be contrasted with the myriads of tiny flashes of light that are seen when the net is first raised. The light in the latter instance is primarily due to copepods and siphonophores and soon dies down leaving only the bright blue luminous spots of *C. serrata.* The color of light was similar, if not identical, to that of living *C. hilgendorfii.* When the plankton tow nets were emptied several times into the bucket, the sea water in the bucket turned brightly luminous. Such sea water, when passed through filter paper, produced light when mixed with *C. hilgendorfii* luciferin. Slight mechanical disturbance of the water in the bucket readily caused *C. serrata* organisms to emit a brilliant blue glow, lighting up the bucket for many seconds. When the water in the bucket was swirled, long trails of blue luminous secretion could be observed.

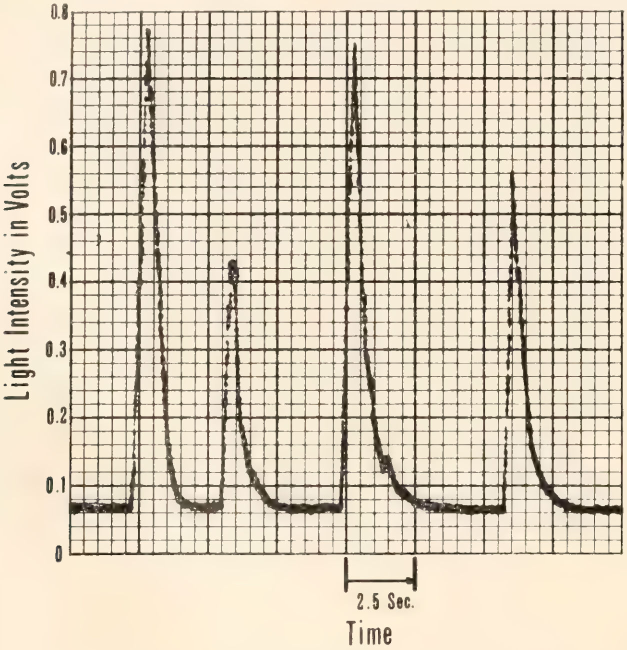

FIGURE 2. Apparent spontaneous flash recordings of 3 *Cypridina serrata* organisms in sea water, recorded with a photomultiplier photometer and strip-chart recorder (Mini-Writer, Watanabe Inst. Co.).

Light response to stimulation was less on moonlit nights. On one moonlit night about 15 individual responses were counted in a 1 hour period, whereas earlier in the month when the moon was dark, thousands of responses could be counted in the same area. On such moonlit nights, several hundred *C. serrata* could still be collected in an evening by towing plankton nets. These organisms were apparently refractory to light stimulation since only a few responses were obtained in the collection area with a flashlight. These organisms also gave almost no streaking in the collecting net and few spontaneous flashes.

2. *Flash recordings*

Collected *C. serrata* did not respond to stimulation by flashlight immediately after collection or later in the laboratory. However, they did emit apparent spontaneous flashes of light which were recorded. A typical set of four flashes is shown in Figure 2. This was made by placing 2 or 4 organisms in a vial containing 10 ml of sea water and monitoring the vial in a photometer. The organisms were isolated with a small glass capillary from a mixture of organisms in a Petri dish with the aid of a low-power microscope. Visual inspection of a vial of *C. serrata* organisms showed the flashes to be either point sources of light, or in some cases, long thread-like streaks or jets of luminous secretion which issued from the organisms. A point source of light was about the size of the body of *C. serrata* so that the whole organism appeared luminous. The light seemed to be of internal origin and did not show any sign of luminous secretion diffusing into the water. Some of the apparent spontaneous flashes were extremely bright, easily visible to the naked eye in a lighted laboratory room whereas some point sources of light were glows that lasted for many minutes (some were watched for over 30 minutes). The flashes in Figure 2 each show a duration of approximately 1.5 seconds. The

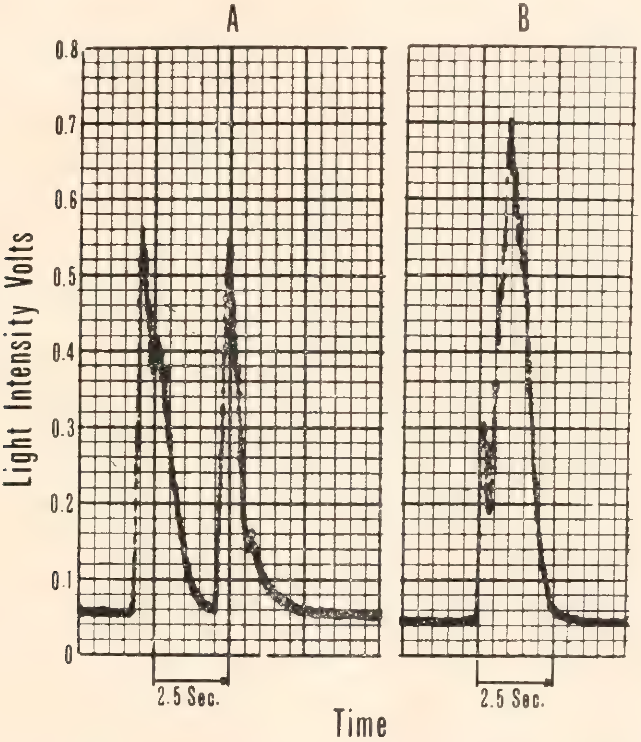

FIGURE 3. Apparent spontaneous flash recordings of 3 *Cypridina serrata* organisms in sea water showing (A) double flash and (B) possibly triple flash (Mini-Writer, Watanabe Inst. Co.).

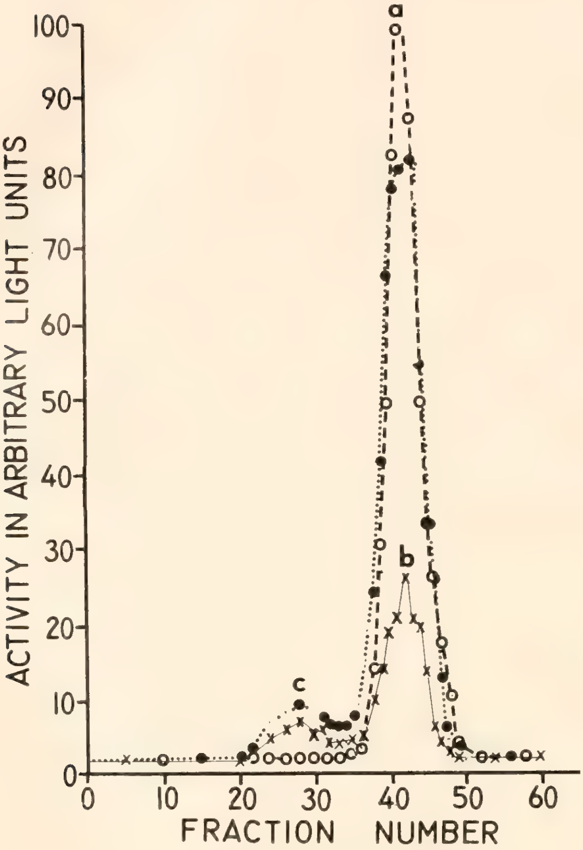

FIGURE 4. Elution patterns of *Cypridina serrata* luciferase (Curve *a*), *Cypridina hilgendorfii* luciferase (Curve *b*), and mixture of *C. serrata* and *C. hilgendorfii* luciferases (Curve *c*) from Sephadex G-200 column. (Note: overlap of some points.)

shape of the flash curve usually varied from those shown in Figure 2 to some with a double spike. The frequency of flashing was irregular and varied with each batch of organisms. The number of active batches, however, was relatively small. Organisms that did not start flashing within 10 minutes after being placed in the photometer usually did not flash later.

A plot of the logarithm of light intensity (Fig. 2) against time shows that the decay for each curve is exponential, with rate constants, from left to right, of 1.43 sec^{-1}; 0.863 sec^{-1}; pre-spike, 1.13 sec^{-1} and post-spike, 0.818 sec^{-1}; and 0.946 sec^{-1}. In the third curve from the left, the exponential decay is interrupted by an after-spike, but the after-spike also decays exponentially. The after-spike appears to be a second flash superimposed on the first. It may be due to triggering in view of the known response of the organism to artificial light. Fig. 3A shows two additional flash recordings with double spikes. Assuming that one animal triggers a second animal, the estimated latency was 500–800 milliseconds for the flash response.

In Figure 3B, which represents still another flash recording, the curve appears to be a superimposition of 3 flashes.

3. *Chromatography of luciferase*

Twelve milliliters of partially purified *C. serrata* luciferase solution possessing a concentration of 0.40 mg/ml and 12 ml of partially purified *C. hilgendorfii* luciferase solution possessing a concentration of 0.25 mg/ml were prepared in

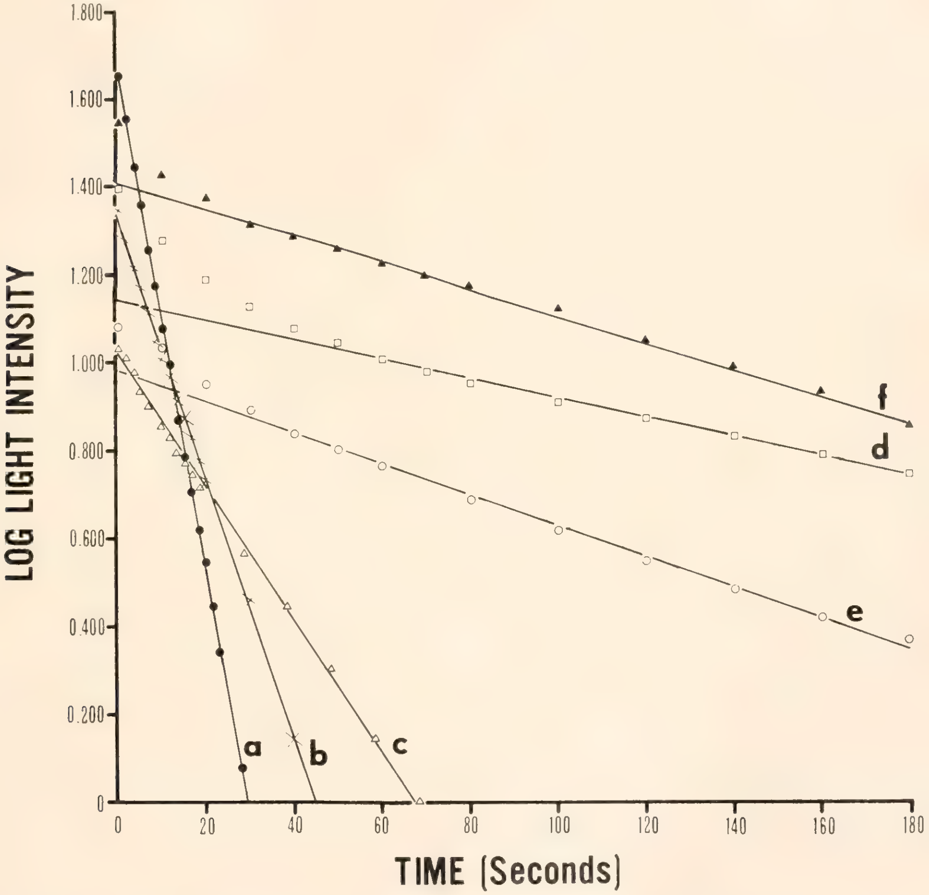

FIGURE 5. Luminescence intensity decay curves of *Cypridina serrata* luciferin-luciferase mixtures, except as noted. For each decay measurement, 1.0 ml of a solution of luciferase dissolved in 0.1 *M* sodium phosphate buffer, pH 6.8, was injected with a hypodermic syringe into a mixture of 1.5 ml of luciferin + 1.5 ml of 0.1 *M* sodium phosphate buffer, pH 6.8, except for curve *e*, in which luciferase was injected into 0.75 ml luciferin + 2.25 ml buffer. Luciferase concentrations: curve *a*, 2.0 mg/ml; curve *b*, 1.0 mg/ml; curve *c*, 0.5 mg/ml; and curves *d* and *e*, 0.133 mg/ml. Curve *f*: 1.0 ml *C. hilgendorfii* luciferase (1.54×10^{-4} mg/ml) injected into 1.5 ml luciferin + 1.5 ml buffer. Curve *e* is shifted upward 0.2 logarithm units for better comparison. Two separate preparations of *C. serrata* luciferin were used: one for curves *a*, *b*, and *c*, and another for curves *d*, *e*, and *f* (Bristol Recorder).

0.07 *M* sodium phosphate buffer, pH 6.8. On assay with *C. hilgendorfii* luciferin, the *C. serrata* luciferase solution possessed a somewhat higher activity than the *C. hilgendorfii* luciferase solution. Four milliliters of each of these preparations were mixed together, the 8 ml remainder of the luciferase solutions being frozen. The 8 ml of mixture were dialyzed 16 hours against the same buffer and put on a 90 cm Sephadex G-200 column. Each eluted fraction (6.3 ml each) was assayed with *C. hilgendorfii* luciferin. One day later the 8 ml of *C. serrata* luciferase solution were thawed, dialyzed as above, and put on the same column. Still another day later, the *C. hilgendorfii* luciferase solution was thawed, dialyzed as above, and put on the same column. The elution curves are shown in Figure 4. All three solutions showed a single activity peak, which appeared at the same place (fraction 42) in the elution diagram. Hold-up volume, measured with 2% dextran blue solution, was 135 ml. Both solutions containing *C. hilgendorfii* luciferase showed small peaks at the beginning of elution due to what might be a polymerized form of the luciferase. We conclude that *C. serrata* and *C. hilgendorfii* luciferases are indistinguishable by gel elution chromatography.

4. *Kinetic measurements*

The decay of light intensity in various mixtures of *C. serrata* luciferin and luciferase was measured. The results are shown in Figure 5, with logarithm of light intensity, which is a measure of reaction rate, plotted against time. In the first experiment, represented by curves *a, b,* and *c,* the concentration of luciferin was held constant and the luciferase concentration was varied so as to give concentrations of C, C/2, and C/4, respectively. In each case, the decay of luminescence followed first order kinetics. The calculated rate constants were 5.67×10^{-2} sec^{-1}, 3.07×10^{-2} sec^{-1}, and 1.52×10^{-2} sec^{-1}, respectively. The corresponding half-times for the decays were 5, 10, and 20 seconds. The rate constants are, therefore, observed to be directly proportional to luciferase concentration. A second experiment, represented by curves *d* and *e,* was run at a lower luciferase concentration. Luciferase concentration was the same (0.133 mg/ml) but the luciferin concentration of *e* was one-half that of *d*. The luminescent reaction initially showed a very high rate of decay, but it soon became first order. The rate constants for *d* and *e* were 1.97×10^{-3} sec^{-1} and 3.34×10^{-3} sec^{-1}, respectively. The rate was thus increased by a factor of 1.70 (rate constant *e*/rate constant *d*) on a one-half decrease in luciferin concentration. The rate constant therefore appears to be dependent on luciferin concentration. Because of a limited supply of *C. serrata* organisms, the experiment could not be carried out with purified *C. serrata* luciferin. However, when *C. serrata* luciferin was replaced with highly purified *C. hilgendorfii* luciferin, the first order rate constants were found to be directly proportional to luciferase concentration and completely independent of luciferin concentration. In the final experiment, *C. serrata* luciferin was run against a single concentration of *C. hilgendorfii* luciferase (1.54×10^{-4} mg/ml). The decay curve, *f*, was typical of the curves previously obtained with a rate constant of 3.00×10^{-3} sec^{-1}. Immediately after mixing, a high initial rate of decay occurred which was followed quickly by a normal first order decay.

5. *Oxygen requirement*

Requirement for oxygen in the luminescent reaction was demonstrated by placing 3.0 ml of *C. serrata* luciferin diluted with 8.0 ml of 0.1 *M* sodium phosphate buffer, pH 6.8, in one arm of a mixing apparatus, and 5.0 ml of *C. serrata* luciferase solution in a second arm. Argon (99.99%) was bubbled through both arms for 12 minutes, then the apparatus was evacuated for 2 minutes with a vacuum pump. The arm containing the luciferin was placed in the cell holder of a photomultiplier photometer, and the luciferase was then added under vacuum. No light was observed. After 17 seconds, air was admitted into the mixture through a capillary tube. The results are shown in Figure 6. The admission of air produced a bright initial burst of light. The high initial rate of decay was rapidly followed by a normal first order decay. The experiment was repeated with *C. hilgendorfii* luciferin and luciferase with the same result. The data indicate that oxygen is required by *C. serrata* in the luminescent reaction.

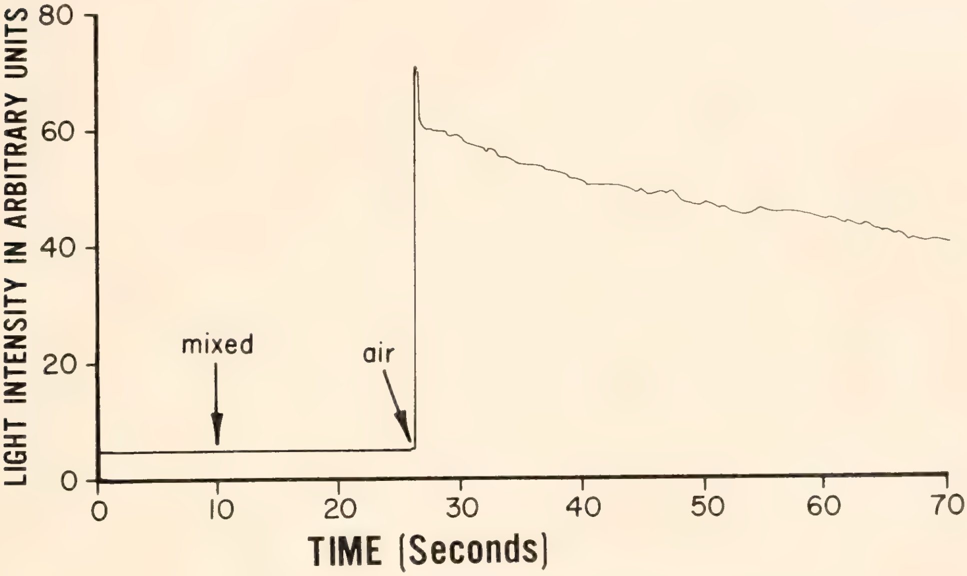

FIGURE 6. Light intensity measurement (tracing of recording) showing requirement for oxygen. *Cypridina serrata* luciferase and luciferin were mixed in absence of oxygen, then air introduced 17 seconds later. Note the high initial rate of decay (Leeds and Northrup Speedomax Recorder).

6. *Inhibition by antibody*

The immunological inhibition experiments are summarized in Table I. Rabbit antibody to *C. hilgendorfii* luciferase was incubated separately with *C. hilgendorfii* and *C. serrata* luciferases, closely matched in activity. After incubation, the residual luciferase activity remaining was determined in the separate mixtures. The antibody is seen to inhibit *C. hilgendorfii* luciferase to a far greater extent than *C. serrata* luciferase. We conclude that *C. serrata* luciferase, while possessing similar catalytic activity, is immunochemically different from *C. hilgendorfii* luciferase.

7. *Chromatography of luciferin*

Paper chromatography of *C. serrata* and *C. hilgendorfii* luciferins gave identical R_f values (average of 0.65). We conclude that the luciferins are very similar, if not identical.

DISCUSSION

According to Harvey (1952), the genus *Cypridina* includes between 20 and 25 species. Among these, two species, *Cypridina hilgendorfii* and *C. noctiluca,* are known to be luminous. The luminescence of *C. hilgendorfii* has been extensively studied and needs little comment. *C. noctiluca* has been studied on different occasions by Haneda (1940, 1953, 1955). In addition, *Cypridina norwegica* is reported by Harvey (1952) to yield extracts that give light-emitting cross-reactions with extracts of *C. hilgendorfii.* Two other positive luciferin-luciferase cross-reactions have been reported. A close relation, *Pyrocypris,* a genus belonging to the family Cypridinidae, has been shown by Harvey (1922) to give a luciferin-luciferase cross-reaction with *C. hilgendorfii.* A *Cypridina* species from Jamaica, which emits a bluish yellow or yellowish light, has been found by Harvey (1924) to cross-react with *C. hilgendorfii,* which emits a bluish light. Harvey found that *C. hilgendorfii* luciferase when cross-reacted with Jamaican *Cypridina* luciferin gave a bluish light, whereas Jamaican *Cypridina* luciferase when cross-reacted with *C. hilgendorfii* luciferin gave a yellowish light. The results indicated that the color of light depended on the source of luciferase. More recently, a new

TABLE I

Inhibition of luciferase activity by rabbit antibody

Incubation mixture	*Cypridina hilgendorfii*		*Cypridina serrata*	
	Initial light intensity in arbitrary units	Per cent of control	Initial light intensity in arbitrary units	Per cent of control
Control	42.0†	100	44.5‡	100
Control + 2.0 × 10^{-2}mg antibody*	24.0	57	41.0	92
Control + 6.0 × 10^{-2}mg antibody*	7.5	18	28.5	64
Control + 18.0 × 10^{-2}mg antibody*	2.0	5	15.0	34

* Prepared from pooled antisera of rabbits immunized against *C. hilgendorfii* luciferase.

† Control consisted of 1.54 × 10^{-4} mg of purified *C. hilgendorfii* luciferase dissolved in 1.0 ml of 0.1 *M* sodium phosphate buffer, pH 6.8.

‡ Control consisted of 1.33 × 10^{-1} mg of partially purified *C. serrata* luciferase dissolved in 1.0 ml of 0.1 *M* sodium phosphate buffer, pH 6.8. Note: All mixtures were brought up to 1.5 ml final volume with 0.1 *M* sodium phosphate buffer, pH 6.8, after adding antibody. After 19 hours incubation at 4° C, luciferase activity in a 1.0 ml aliquot was determined as the initial maximal light intensity observed on adding a constant saturating concentration of *C. hilgendorfii* luciferin

luminous species, *Vargula harveyi,* has been reported from Long Bay, northeast Jamaica, by Seliger and McElroy (1965) and described by Kornicker and King (1965).

The present study illustrates the difficulty that may be encountered in trying to identify an organism that luminesces at sea unless specimens are taken. The evidence in the present instance is not unequivocal, but several reasons suggest that the organisms involved at Madang are *Cypridina serrata.* First, there was a direct correlation between the flashlight response and the presence of *C. serrata* in the water. *C. serrata* was absent from areas where the flashlight response was negative. When the plankton net was towed in such areas, the net brought up only mixtures of copepods, non-luminous shrimps, siphonophores and some dinoflagellates. No bright blue luminous spots or *C. serrata* organisms were found in the net. Second, the blue color of the luminous cloud was similar to the color of light produced by *C. serrata* in the laboratory. This evidence, however, cannot be considered very strong since the color of luminescence of most marine organisms is bluish. Third, the cloud was observed to drift in the water during the 3–4 seconds of decay. The size of the cloud was many times greater than any of the luminous organisms collected in the water. These observations are difficult to explain except under conditions in which luminous substances or components that react to produce light are ejected into sea water and afterwards diffuse or are carried by the current. Fourth, the behavior of these organisms toward moonlight was similar to *C. hilgendorfii.* On moonlit nights, few organisms were found to respond to light stimulation and only a relatively small number appeared in the water. In Japan, the authors have also observed that *C. hilgendorfii* organisms appear in smaller numbers on moonlit nights.

Another question which naturally arises concerns the origin of luciferin and luciferase in the extracts studied. The freeze-dried material contained both *C. serrata* and a mixture of luminous and non-luminous copepods, two closely related ostracods. The luciferins and luciferases from these organisms could possibly give light-emitting cross-reactions with each other and with the luciferin and luciferase of *C. hilgendorfii.* However, careful studies conducted by Harvey (1926) seem to rule out this possibility. Harvey showed that the hot- and cold-water extracts of copepods do not give the luciferin-luciferase reaction with each other and do not cross-react with the extracts of *Cypridina.* Thus, the luciferin and luciferase in this study appear to be of *C. serrata* origin.

The response of *C. serrata* to light stimulation is strikingly similar to that of *C. noctiluca,* observed by Haneda (1940). The mechanism whereby a luminous cloud is produced by *C. serrata* is undoubtedly similar to that in *C. hilgendorfii.* In the latter, luciferin and luciferase are ejected into the water from storage glands, where on diffusion, the light reaction takes place. The elucidation of the light-sensitive response mechanism awaits future study. The finding of the luminescence response system in *C. serrata* may be considered significant since light-stimulated luminescence is unknown except for *C. noctiluca* and fireflies exposed to species-specific flash patterns. Turner (1966), however, states that numerous reports by mariners exist in which luminescence has been observed when light is shone into the sea.

C. serrata is stimulated to luminesce either mechanically or by artificial light and, in addition, luminesces spontaneously; but *C. hilgendorfii* is known to be stimulated only mechanically. Although *C. serrata* did not respond to flashlight stimulation when once placed in the collection bucket, it did respond when the water was agitated with the hands. The non-responsiveness of captive *C. serrata* to flashlight stimulation was not further investigated and, therefore, no explanation can be given for the lack of response.

The flash recordings of isolated *C. serrata* organisms in Figures 2 and 3 suggest that the flash of one organism (produced either spontaneously or by collision with the wall or another organism) triggers a neighboring organism to flash. This assumption is reasonable because of the known response of *C. serrata* to artificial light and by the fact that a flash pattern containing an after-spike is not normally observed with luminous organisms. No comparisons can be made between the *C. serrata* flash and the *C. hilgendorfii* flash, as no records exist, but the dinoflagellate *Noctiluca* flash has been investigated by Nicol (1958), Hastings (1959), and Eckert (1965, 1967). These results show a flash duration of about 100 milliseconds, a latency of 2–5 milliseconds, and a mean rate constant for the exponential decay of luminescence of 0.088 milliseconds^{-1}. Compared to the *C. serrata* flash, the flash duration is about 15 times shorter, the latency is about 200 times shorter, and the decay rate is approximately 100 times faster. Data on the copepod flash, however, are not available in the literature and no comparison can be made. Since the duration of a flash depends on factors such as mixing and concentration of reactants, it would be difficult to compare a recorded flash with a light-stimulated flash in the ocean, and with light emission from a mixture of luciferin and luciferase.

The luminous cloud, the thread of luminous secretion, and the point source of light, all produced by *C. serrata,* indicate that the organism is able to control luminescence. It is possible that the quick ejection of a cloud of luminous material serves to propel the organism through the water and provide a screen to escape from a predator. The production of a point source of light appears to indicate a mixing of luciferin and luciferase within the organism or mixing just at the gland orifices through a finely regulated release of luciferin and luciferase. The ability of *C. serrata* to control its luminescence would account for the luminous clouds observed in the sea and the flashes observed in the laboratory. When collecting *Vargula harveyi* at Long Bay, Jamaica, in 1965 and 1967, Dr. Howard Seliger (personal communication) of the McCollum-Pratt Institute, Johns Hopkins University, observed point source of luminescence similar to ours. The whole body of *V. harveyi* was luminous without any appearance of luminous secretion and he believes that the mixing of luciferin and luciferase takes place by one of the above two mechanisms.

In the reaction between crude *C. serrata* luciferin and partially purified luciferase, the decay rate is initially very high. This anomaly was first observed by Amberson (1922) in the *C. hilgendorfii* luciferin-luciferase reaction. In his early experiments, mixing *C. hilgendorfii* luciferin and luciferase resulted in a bright initial flash of light, corresponding to a high initial rate of decay, which was then followed by a normal first order decay. The bright initial flash was attributed by Amberson (1922) to the active site of the enzyme being free of luciferin at the outset.

If *C. serrata* luminescence is due to a first order reaction, theoretically the rate constant should be independent of luciferin concentration. However, a near doubling of the rate constant occurs when the luciferin concentration is halved (Fig. 5d and e), indicating that the rate constant is dependent on luciferin concentration. The rate constant, however, is independent of luciferin concentration when highly purified *C. hilgendorfii* luciferin is used. A similar result has also been noted in the *C. hilgendorfii* luminescent reaction. Amberson (1922), Stevens (1927), Harvey and Snell (1931), and Chase (1956) observed that the first order rate constant increased as the initial luciferin concentration was decreased. The rate constant increase was about two-fold in the studies of Amberson (1922) and Chase (1956) and five-fold in the experiments of Harvey and Snell (1931). Subsequently, Chase and Harvey (1942) found that the rate constant showed no change with luciferin concentration if highly purified luciferin and partially purified luciferase were used. They concluded that some impurity in either luciferin or luciferase was responsible for the increase in the rate constants of the earlier workers. In the present work with *C. serrata,* the result obtained by using purified *C. hilgendorfii* luciferin appears to indicate that the increase in the rate constant is due to an impurity in the *C. serrata* luciferin.

Three other aspects of the study require brief comments. The data (Fig. 6) show clearly that the luminescent reaction of *C. serrata* requires oxygen. The same requirement for oxygen was demonstrated many years ago for the *C. hilgendorfii* reaction by Harvey (1917, 1920). The immunochemical results (Table I) show that *C. serrata* luciferase is a related but distinct enzyme from *C. hilgendorfii* luciferase. Tsuji and Haneda (1966) have previously shown that antibody to *C. hilgendorfii* luciferase may be used to distinguish *C. hilgendorfii* luciferase from another closely related luciferase. Finally, the identical R_f's show that *C. serrata* and *C. hilgendorfii* luciferins are very similar, if not the same.

We thank the following individuals for their generous assistance: Mr. Robert Gibson, Territory of Papua and New Guinea, Department of Agriculture, Stock and Fisheries, Madang, for providing a boat and operator employed in the collection of *Cypridina* organisms; Dr. J. Woodland Hastings, Harvard University, for the loan of the photomultiplier photometer; Dr. Louis S. Kornicker, Smithsonian Institution, for identifying the *Cypridina* specimens and for discussions on Ostracoda; and Dr. John B. Buck, National Institutes of Health, for comments on the flash recordings.

SUMMARY

1. The physical appearance and bioluminescence behavior, and light-emitting reaction of the marine ostracod crustacean, *Cypridina serrata,* are described.

2. In the natural environment of the sea, the free-swimming *C. serrata* appears to emit almost instantaneously a bright blue luminous cloud when stimulated with artificial light.

3. The method of light production, consisting of the ejection of luciferin and luciferase into sea water, and the color of light are similar to that of *C. hilgendorfii.*

4. In captivity, *C. serrata* emits apparent spontaneous flashes of light, whose duration is approximately 1.5 seconds, with an apparent latency of 500–800 milliseconds.

5. *C. serrata* luciferase cannot be distinguished from *C. hilgendorfii* luciferase by gel elution chromatography but may be distinguished immunochemically.

6. The luminescence of *C. serrata* is due to a first order reaction, similar to that of *C. hilgendorfii.* The luciferins and luciferases of both organisms cross-react to give light.

7. The luminescence of *C. serrata,* like *C. hilgendorfii,* is oxygen dependent.

8. *C. serrata* luciferin is similar, if not identical, to *C. hilgendorfii* luciferin when compared by paper chromatography.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY
1970

ABSTRACTS OF SEMINAR PAPERS

JULY 14, 1970

Interpopulation variation in shell components in the stream limpet, Ferrissia rivularis. W. D. RUSSELL-HUNTER, ALBERT J. BURKY AND R. DOUGLAS HUNTER.

In freshwater snails—as in the majority of molluscs—the secreted shells have two principal components: a meshwork of hardened protein and crystalline calcium carbonate (the latter secreted after active uptake directly from environmental water). Earlier work in Scotland showed that in the euryoecic species, *Lymnaea peregra,* the thickness (and mass) of the calcareous shell varies with the calcium available in the waters. Thus, *Lymnaea* expends about the same energy on shell-making no matter what the environmental hardness. The case of *Ferrissia* in natural creek populations of upstate New York is strikingly different. As we have reported elsewhere (*Science,* **155**: 338-340, 1967), although these creeks vary in dissolved calcium from 10.4 mg/l (Black Creek) to 67.6 mg/l (Limestone Creek), the highly significant differences in shell calcium from 82 mg/g live weight (Fish Creek) to 145 mg/g (Canandaigua Outlet) are not correlated. Anabolic concentration ratios ranged from 1609:1 to 10,615:1 and there was other circumstantial evidence of physiological races.

Recently we have measured total organic carbon and total nitrogen in shells of limpet growth stages from these populations. The noncalcareous component is obviously mostly protein because the **C**:**N** ratios are consistently about 3.25:1. Organic carbon in μgC per standard shell (3.5 mm) ranges from 7.0 (Canandaigua Outlet), 8.4 (Limestone Creek), 8.7 (Slocum Creek), 9.4 (Fish Creek), 10.2 (Big Bay Creek), 10.3 (Chittenango Creek), to 20.8 (Black Creek). Ranking the protein content of the shells from these seven creeks (in the above order) from lowest 1 to highest 7; and similarly ranking dissolved calcium of the creek waters from lowest A to highest G, and shell calcium from I–VII; we have the following sets (from Canandaigua to Black Creeks): 1-F-VII, 2-G-III, 3-B-II, 4-C-I, 5-D-VI, 6-E-V, and 7-A-IV. Thus there is neither an inverse relation between shell protein and calcium (expected by an "adaptive value" hypothesis) nor any direct relationship (expected if both relate to levels of energy turnover). It should be noted that in content of shell protein and of shell calcium (as even more clearly in other measurable characters), the variation *within* the majority of single populations is very much *less* than the range of variation for the species as a whole. It seems that genetic controls of shell secretion for the two major components are independent, and that the chances of genetic dispersal among the isolated creek populations of this limpet have resulted in some rather inappropriate shells in certain habitats.

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A fundamental error in the general model of genetic selection. ROGER MILKMAN.

The general selection model states that the relative survivorship of alternate genotypes is independent of frequency. For example, the proportion of *aa* zygotes surviving to reproduce (number of *aa* adults/number of *aa* zygotes) is said to be in a constant ratio to the proportion of *Aa* zygotes surviving to reproduce. The proportion surviving may change during selection;

the ratio of these proportions does not. (All conditions are stipulated to remain constant with the exception of the genotype frequencies, which change during the course of selection.)

In constructing a realistic model based on elementary breeding procedures, we find that this ratio is not independent of genotype frequency. For example, consider two genotypic classes, *aa* and *Aa*, each normally distributed for bean weight with means of 8 and 9 grams, respectively, standard deviation being 1 gram in each case. These distributions (reflecting the impact of additional gene loci and of environmental factors) overlap, and both classes are likely to be represented in the heaviest 5% of the beans, which are chosen for seed. But as the *Aa* class increases in frequency, the competitive standard rises—beans must be heavier to make the grade. Mathematically, each common selection threshold is associated with a unique ratio: the proportion of *aa* zygotes that survive divided by the proportion of *Aa* zygotes that survive, *e.g.*, $378/9462 \div 122/538 = 0.176$. It can be shown that as the frequency of a superior genotype increases, the *relative* performance of an inferior genotype (*cf.* the general selection model's *adaptive value*, or W) decreases, and this decrease is substantial for a large range of values.

Changes in selection intensity also cause changes in relative survivorship; the use of dissimilar standard deviations in the example above would not alter the results materially.

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Ultrastructure of the negatively stained spermatozoon of the earthworm. CATHERINE HENLEY.

Spermatozoa from the seminal vesicles of *Lumbricus terrestris* were negatively stained with 1% PTA, pH 6.8, for 4–10 min, and examined with the Zeiss EM 9A. The preparations had large numbers of intact spermatozoa, in which many details of the structure of acrosome complex, nucleus, mitochondrial middle piece and axoneme closely paralled similar features shown in sectioned material by Anderson and Ellis, and others. In acrosomes interpreted to have undergone a partial acrosome reaction, the acrosome vesicle and rod looked very much like those seen in sections, but the material inside the vesicle had a coiled appearance, rather than a uniform one, and there was always an accumulation outside the acrosome tip of similar-appearing material. In unreacted acrosomes no internal substructure could be discerned and there was never material at the tip. In the 9 + 2 tail, the microtubules usually fell in rather straight or gently curved configurations, and the singlets were in such close proximity to one another as to appear as one structure. Beta glycogen granules, recently described by Anderson and Personne in sectioned earthworm spermatozoa, were associated with the doublets. Diastase treatment resulted in the disappearance of these granules, but 3-hr incubations of spermatozoa in distilled water at room temperature had no effect on them. However, such prolonged distilled water treatments caused the doublets to fall on the supporting Formvar film in tight coils, while the singlets (usually still attached to one another along most of their lengths) pursued very different courses, along jagged paths with sharp bends, and for long distances. They often assumed remarkably symmetrical arrow-shaped configurations. The cross-connections between the singlets occurred at regular intervals of *ca.* 150 Å and were apparently very strong, as evidenced by the close adherence of the singlets to one another even through turns of 20° or less.

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Hyaluronate and hyaluronidase in the blastema of the regenerating newt limb. BRYAN TOOLE AND JEROME GROSS.

Release of dedifferentiated cells from the structural tissues of the stump and accumulation of these cells in an outgrowing blastema are essential for regeneration of an amputated newt limb. This process requires extensive proliferation and migration of the blastema cells and retention of their dedifferentiated state for a period of approximately two weeks in the case of the adult newt, *Diemictylus viridescens,* studied here.

A very marked enhancement of the incorporation of radioactive precursors into hyaluronate has been found in the early blastema as compared to the normal unamputated limb. At 10 days of regeneration this uptake is most active in the most distal portion of the regenerate but there is a gradation of degree of uptake from zero to 40-fold along the length of the stump. Between 15 and 20 days of regeneration, when the first sign of differentiation of precartilage has appeared in the blastema, there is a drop in the uptake of isotope into hyaluronate but a rise in uptake into chondroitin sulfate, a component of cartilage. These changes continue during the following 30 days of regeneration, and concomitant with them hyaluronidase activity appears in the blastema. This enzyme cannot be detected prior to 15 days or after 50 days of regeneration nor in the normal unamputated limb.

It appears, therefore, that the extracellular phase of the early dedifferentiated blastema is rich in hyaluronate and that this macromolecule is removed at the onset of precartilage differentiation.

This investigation was supported by a grant from the National Science Foundation.

Reversible inhibition of RNA phage replication and macromolecular synthesis by levorphanol. ERIC J. SIMON, DAVID J. GARWES AND JUDITH RAND.

Levorphanol inhibits the production of infectious RNA phages MS2 and $Q\beta$ by more than 99 per cent. Even at concentrations at which effects on bacterial growth are minimal, phage production is decreased by 85–90 per cent. When the drug is washed away 30 or 60 minutes after infection, phage multiplication begins almost immediately and a normal yield of infectious particles results. Levorphanol is most effective when added before or at the time of infection, but becomes less so with increasing intervals between infection and drug treatment. When added 30 minutes or more after infection levorphanol has no effect on plaque formation. Studies on phage RNA and protein synthesis yielded results similar to those on phage multiplication. When levorphanol is added simultaneously with phage there is no detectable phage RNA nor protein synthesis, while levorphanol addition 30 minutes later results in normal macromolecular synthesis. An alteration in bacterial membrane permeability resulting in exclusion of the drug late in the life cycle of the phage has been ruled out. An effect of levorphanol on an early event in phage reproduction is postulated.

Supported by Grants MH-10227 and AM-01845 from the National Institutes of Health. Eric J. Simon is a Career Scientist of the Health Research Council of the City of New York.

Hydranth regulation in the colonial marine hydroid Podocoryne carnea. MAX BRAVERMAN.

Unlike the many highly regular colonial hydroids, *Podocoryne carnea* shows no obviously visible regularity in the distribution of polyps on the stolons. Since the stolons are completely adherent to the substrate, in this case a microscope slide, an exhaustive description of the colony morphology is contained in a photograph. Analysis of daily photographs of growing colonies shows that statistical regularity does obtain: the number of hydranths and the total length of stolon in a colony tends to constant proportionality.

To determine if this proportionality results from feedback control of young polyp formation, the initiating polyps were removed from young colonies before any new polyps had formed. Removal of the initiating polyp stimulates the formation of new polyps within three days, compared to fed or starved unoperated controls. The difference is much greater, however, between operated and fed controls. A significant difference in hydranth formation between fed and starved controls implicated starvation as a stimulator of polyp formation.

To evaluate the influence of polyp removal independently of that of starvation, polyps were removed from one half of a colony and the colony fed and photographed for six successive days. Since stolon growth on the side from which polyps were removed was the same as on the control side, it could be assumed that the food captured by the control side was equitably distributed and the requisite separation effected. Under these circumstances, a significant difference ($P = 0.007$) between the number of polyps forming during the two days following removal could be seen. About two times as many polyps formed on the side from which the polyps were removed as on the unoperated control side, indicating localized feedback control of hydranth formation.

July 28, 1970

Excitatory and inhibitory regulation of efferent nerve activity in the phallic nerve of the cockroach Periplaneta americana (L). M. E. Spira, I. Parnas and F. Bergmann.

Phallic nerve activity was recorded with suction electrodes during electrical stimulation of the ventral nerve cord (V. N. C.). In all preparations a train of impulses delivered to the V. N. C. caused up to 600% increase in phallic nerve firing rate for periods of up to 10–15 sec. The increased firing rate is dependent on the stimulus intensity, train duration, and stimulus frequency. In a few cases a single pulse was sufficient to cause the increase in firing rate. In these cases the firing rate increased with stimulus intensity. Stimulation of both ipsi and contralateral connectives caused activation of the phallic nerve. However the response to ipsilateral stimulation lasted longer and showed a higher frequency. In some preparation a train of stimuli delivered to the V. N. C. induced a silent period. Application of Eserine (mg/ml) to ganglia of the V. N. C. other than the last abdominal ganglion (A6), did not affect the phallic nerve firing rate. However, application of Eserine to the A6 ganglion caused an immediate increase in the firing rate which is blocked after 3 minutes. Application of d-tubo curarine to the preparation did not alter the spontaneous activity. However under these conditions a train of stimuli delivered to the V. N. C. caused a long lasting silent period (10–20 sec). Application of picrotoxin (mg/ml) to ganglia other than A6, caused no change in phallic nerve activity. Only when picrotoxin was applied to A6 could an increase in phallic nerve activity be observed. This increase persisted for 15 minutes. Later the activity decreased gradually to the normal level. At this stage a single stimulus at the V. N. C. resulted in a burst of activity in the phallic nerve. We suggest that the minimal neuronal network to explain the present results involves spontaneously active phallic motoneurons on which excitatory and inhibitory inputs converge from both the ipsi and contralateral connectives.

Electrical and photochemical signs of adaptation in the retina of the skate, Raja erinacea *and* R. oscellata. J. E. Dowling and H. Ripps.

The skate retina appears histologically to contain only rod photoreceptors and should provide, therefore, an ideal tissue for studying the response properties of the scotopic mechanism. In order to determine whether the b-wave of the electroretinogram (ERG) provides a valid index of retinal sensitivity, b-wave and ganglion-cell thresholds were compared during equivalent stages of light- and dark-adaptation. Under all conditions tested, both responses exhibited remarkably similar alterations in sensitivity. For example, on exposing the retina to a moderately bright background illumination there was a period during which neither response could be elicited with intense test flashes. The duration of this silent period depended upon the intensity of the steady background, and lasted for as long as 20 minutes when a significant fraction of the rhodopsin was bleached. However, once retinal excitability returned, thresholds continued to fall until a stable level was reached; at these levels, the increment threshold was a linear function of background illuminance.

Threshold changes in the ERG and ganglion cell discharge during dark adaptation also followed a parallel time course. After a flash bleach which denuded the retina of about 80 per cent of its rhodopsin content, neither response could be elicited for 10–15 minutes. But following this period of electrical silence, thresholds fell rapidly to within 3 log units of the dark-adapted level. Further recovery of sensitivity was slow, requiring an additional 90 minutes in darkness to reach completion. Fundus reflectometric measurements of rhodopsin regeneration enabled us to correlate changes in pigment concentration with the recovery of threshold that occurred during the slow phase of dark adaptation; a linear relation was obtained between rhodopsin concentration and log threshold.

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The role of the sodium pump in adaptation in the frog muscle spindle. DAVID LANDOWNE.

The mechanism of sensory adaptation in muscle spindles has not been demonstrated previously although visco-elastic models have been proposed whereby the effective stimulus is reduced. Another possible method of reducing the rate of firing of a nerve cell is to hyperpolarize the cell. This could occur *in vivo* by the action of an electrogenic sodium pump.

When a muscle spindle is rapidly stretched and held at a new constant length a train of impulses occurs. If the instantaneous frequency (reciprocal of the interspike interval) is plotted semilogarithmically against time three phases of adaption may be seen. The first phase is the classical dynamic response and the next two are included in the static response. Removing external potassium, adding 10 μM strophanthidin, or replacing external sodium with lithium all selectively and reversibly block the second phase. As these three conditions would also be expected to block the sodium pump it is concluded that this phase should be closely associated with the sodium pump, which presumably hyperpolarizes the cell in response to the sodium which enters the cell during the generator and action potentials. The observation that it is the second phase which is inhibited supports the division into three phases.

Ultrastructure of the secondary septa of Metridium *sp.* WALLIS H. CLARK, JR. AND GERTRUDE W. HINSCH.

The secondary septa of male *Metridium* sp., collected at Woods Hole during late July, were examined with both the light and electron microscopes. The endoderm of the basal portions of the septa consists of tubular aggregates of spermatogenic tissue surrounded by occasional interstitial cells. The endoderm is separated from the ectoderm by the mesoglea. The ectoderm consists of several cell layers, the outermost layer being composed of epithelial cells which face the gastrocoel. The majority of the cells found between the outer epithelial layer and the mesoglea contain homogeneous, secretory-like inclusions. In some instances cells filled with such inclusions appear to be secreting their cytoplasmic contents into the gastrocoel. The epithelial cells contain large Golgi bodies and large, membrane bound aggregates of flocculant material of varying electron density. Septate desmosomes are commonly found between the limiting membranes of these cells. Each of these epithelial cells possesses a single flagellum, the base of which is associated with two centrioles and adjacent cytoplasmic rootlets. The flagella extend from the cell surface and are surrounded by a very fine, electron dense, fibrillar material. Closely associated with this fibrillar material are long undulating microvilli. There are usually 9 microvilli, though some variation in number, 8 to 10 have been noted.

The function of these flagellar-villus aggregates is unknown; however, it seems likely that they may be sensory in nature due to their similarity to the flagella of nematocytes and the sense hairs described in *Hydra*.

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AUGUST 4, 1970

Metabolic controls and biological variation. NEAL W. CORNELL.

Although the occurrence of intraspecific physiological variation has been widely recognized, there appears to be few studies and little understanding of the sources of such variation. Consequently, an investigation was undertaken concerning possible enzymatic sources of continuous variation in mammals, *i.e.*, the type of variation seen in parameters like growth rate during maturation, basal metabolic rate, and longevity.

From evolutionary considerations it seems unlikely that genetic differences in the catalytic sites of enzymes could account for widespread variation. It is proposed, however, that genetic differences in regulatory properties of enzymes sensitive to feedback control might be more common in wild populations and could substantially alter overall patterns of metabolism. The proposal is based in part on studies which show that such differences can be induced with chemical mutagens, resulting in viable organisms with significantly altered physiology. Theoretically, the redundancy of controls in, for example, carbohydrate metabolism should make mutations that affect regulatory properties of enzymes more permissible. To illustrate, citric acid, a component of the Kreb's cycle and also the initial substrate for fatty acid biosynthesis, acts as an allosteric effector for three different enzymatic reactions. The summed effects of citric acting as an allosteric agent is to diminish oxidation of carbohydrates and enhance the biosynthesis of fatty acids (storage) under conditions of high ATP concentration.

The consequences of altering the regulatory properties of acetyl-CoA carboxylase, one site affected by citrate, have been evaluated in a computer simulation study. A very small (5-fold) decrease in the K_m of acetyl-CoA carboxylase for citrate would result in marked increase in the biosynthesis of fatty acids relative to oxidative metabolism. Experiments will be conducted with defined strains of mice to evaluate the predictions of the simulation model.

Supported by grant HDO3620 from the National Institutes of Health.

Analysis of the color changes induced by serotonin (5-hydroxytryptamine) and lysergic acid diethylamide (LSD) in the fiddler crab, Uca pugilator. MILTON FINGERMAN AND K. RANGA RAO.

Serotonin in dosages of 0.1 μg and above per crab evoked pigment dispersion in the erythrophores of eyestalkless and intact *Uca*. Similar dosages of LSD evoked pigment concentration in the erythrophores of intact but not eyestalkless *Uca* (light patch variant from Panacea, Florida). LSD and serotonin were ineffective *in vitro* when assayed on legs isolated from *Uca* showing that they do not directly stimulate the erythrophores. In contrast, red pigment-dispersing and pigment-concentrating polypeptide hormones from the eyestalks were effective both *in vitro* and *in vivo*. Assay of extracts of supraesophageal ganglia with the attached circumesophageal connectives from serotonin-injected crabs revealed a marked decrease in the red pigment-dispersing potency of such extracts compared with controls, indicating that serotonin may have stimulated release of a red pigment-dispersing substance from the neuroendocrine system. In an effort to determine the mode of action of LSD, blood from LSD-treated crabs was assayed for erythrophorotropic activity on eyestalkless crabs. It was found that blood from LSD-treated crabs on a black background evoked more red pigment concentration than did that of the controls. Control crabs on a black background normally contain more red pigment-dispersing hormone than pigment-concentrating hormone. It appears that LSD shifted the hormone balance in the blood in favor of the red pigment-concentrating hormone by either inhibiting the release of red pigment-dispersing hormone or by stimulating the release of red pigment-concentrating hormone. The fact that LSD antagonized the action of serotonin but not that of the red pigment-dispersing polypeptide lends strong support to the view that LSD most likely acts by inhibiting the release of red pigment-dispersing hormone from the neuroendocrine system of the crab. The present studies and the finding of serotonin in the nervous system of *Uca* (Spirtes and Fingerman, unpublished data) indicate that this indolealkylamine may be normally involved in the control of color changes in this crab.

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Enzymatic hydrolysis of the nerve gases DFP and Tabun in relation to nerve function. FRANCIS C. G. HOSKIN.

The organophosphorus cholinesterase inhibitor, diisopropylphosphorofluoridate (DFP), is hydrolyzed by an enzyme in squid axons and especially in squid head ganglia called "DFPase" with a $K_m = 6.25 \times 10^{-3}$ *M*. Another organophosphorus cholinesterase inhibitor, ethyl N,N-dimethylphosphoramidocyanidate (Tabun), is hydrolyzed only slowly by squid nerve. At 10^{-2} *M* the rates are 750 ± 9 μmoles DFP and 83 ± 18 μmoles Tabun hydrolyzed per gram head ganglion per hour. The situation is reversed in rat serum, the rates being 11 ± 1 μmoles DFP and 77 ± 10 μmoles Tabun per milliliter serum per hour. The relative rates of hydrolysis of DFP, of Tabun, and of mixtures of the two by squid nerve indicate that the two substrates compete for a common site on a single enzyme. When a squid axon is bathed in 10^{-3} *M* Tabun, this compound penetrates in its active form, attaining about 50% of the outside concentration in 1 hour. This is because, in contrast to DFP, Tabun is only slowly hydrolyzed by squid nerve while, like DFP, Tabun is lipid-soluble. Furthermore, a solution of cholinesterase is completely inhibited by DFP at about 10^{-6} M, but by Tabun at about 10^{-7} M. With these properties in mind, conduction was measured in squid axons bathed in Tabun or DFP in buffered sea-water. DFP blocks conduction in an intact axon at 5×10^{-3} M, whereas Tabun at 10^{-2} M does not block conduction even after a 1-hour exposure to the compound. These results raise the question whether acetylcholinesterase in the conducting membrane is essential for axonal conduction, and suggest that the block obtained with the high concentrations of DFP may have been due to acid formation on enzymatic hydrolysis of DFP.

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Chemistry and biology of nemertine neurotoxins. WILLIAM R. KEM.

Bacq reported the existence of two neurotoxins, "amphiporine" and "nemertine," in the nemertine phylum. Both toxins produced convulsions when injected into crustaceans. In addition "amphiporine" also elicited a nicotine-like contracture of the frog rectus abdominis. A toxin was recently isolated from the hoplonemertine, *Paranemertes peregrina,* and unequivocally identified as anabaseine, 2-(3-pyridyl)-3,4,5,6-tetrahydropyridine. Anabaseine possesses the convulsant and nicotinoid properties of "amphiporine," but has no effect on either the resting and action potentials of the crayfish median giant axon or the compound action potential of the lobster walking leg nerve. The heteronemertine *Lineus ruber* contains two neurotoxic polypeptides corresponding to Bacq's "nemertine." The polypeptides were partially purified by methanol-acetic acid (95:5) extraction and G-25F Sephadex chromatography (0.1 M ammonium acetate-acetic acid, pH 5.0). Crayfish paralytic activity eluted at 1.4 Ve/Vo and 1.7 Ve/Vo. The polypeptides prolong the repolarization phase of the action potential in the lobster walking leg nerve bundle, but lack nicotinic (frog rectus) and hemolytic (human red cells) properties.

Both hoplonemertine (anabaseine) and heteronemertine (polypeptide) toxins are located in the body wall and proboscis integument. Mucus collected from irritated individuals of these species is highly toxic. Presumably these toxins are chemical defences against potential predators.

AUGUST 11, 1970

Mechanisms of lysosomal hydrolase from phagocytic cells. GERALD WEISSMANN, PETER DUKOR AND ROBERT ZURIER.

Since Metchnikoff, it has been appreciated that phagocytic cells release biologically active substances into surrounding tissues when they engage foreign material. To study mechanisms of enzyme release during phagocytosis *in vitro,* purified mouse peritoneal cells and polymorphonuclear leucocytes from human blood were exposed to particles (zymosan, latex, opsonised sheep erythrocytes, aggregated albumin). Uptake of undigestible, but not of digestible, materials was associated with release (up to 15% in 2 hrs from macrophages; up to 25% in 30 min from polymorphs) of lysosomal enzymes: β-glucuronidase, aryl sulfatase, acid phosphatase, and cathepsin D; no release of cytoplasmic lactase dehydrogenase was observed during phagocytosis, although freezing or mild sonication readily released this marker. Hydrolase release did not depend upon loss of cellular integrity as judged by dye exclusion or by viability of macrophages in long term cultures. Agents which elevate the level within cells of cyclic AMP (cyclic 3′5′ adenosine monophosphate) retarded (1) extrusion of acid hydrolases (2) uptake of heat aggregated bovine serum albumin (3) degradation of previously ingested heat aggregated bovine serum albumin. Each of these activities could be duplicated by exposing phagocytes to colchicine or vinblastine. The agents which were effective were dibutyryl-cyclic AMP, cyclic AMP with theophylline, prostaglandin E_1 with theophylline *etc* but these needed to be present at concentrations above 10^{-5} M. At lower concentrations cyclic nucleotides markedly enhanced particle uptake and degradation. These studies indicate that cyclic AMP (at pharmacologic concentrations) and microtubule reagents can act to block the merger of lysosomes with early phagocytic vacuoles, and furthermore, that their site of action is similar or identical.

Isoelectric focusing of lens gamma (γ) *crystallins.* S. ZIGMAN.

By isoelectric focusing in a pH gradient, the seven components or isomers of rat lens γ-crystallin have been isolated. In 6M urea, the isoelectric points obtained ranged from pH 8.9 to 7.3. All constituents were identical immunologically, and similar in molecular weights (16–18,000), ultraviolet light extinction ($E_{1\text{ per cent}}^{1\text{ cm}} = 18\text{–}23$), relative fluorescences, N-terminal group (alanine), and content of hydrophobic amino acids and ½ cystines. The content of charged amino acids (lysine, arginine, aspartic and glutamic acids) differed appreciably. Differences in the exposure of charged side chains could account for the different isoelectric points found. The components with higher isoelectric points were rich in —SH groups (2–3 moles/mole protein), while those with lower isoelectric points contained 2 —SS— bonds. When total γ-crystallin was oxidized in H_2O_2, all —SH containing material was precipitated, but the lower isoelectric point constituents remained soluble.

Between 1 and 52 weeks of age, a marked fall in the levels of the three γ's with higher isoelectric points and an increase in the four with lower isoelectric points was found. The incorporation of C^{14}-amino acids *in vitro* into those γ's decreasing in level was diminished, while for those increasing, it was increased. Wearing out or stimulation of long-lived M-RNA molecules known to be present in the lens may regulate this change. γ's with higher isoelectric points and —SH constant were predominant in the rat lens nucleus (the oldest portion) where precursors of the urea insoluble (or —SS— linked) protein increase with aging.

Isoelectric focusing profiles of rat, dogfish, cow and human lens γ-crystallins were compared. For dogfish, cow and human γ's, four components each were found. Isoelectric points for the γ's of rats ranged from pH 8.9 to 7.3; of dogfish from pH 8.8 to 7.2; of cow from pH 8.1 to 7.4, and of human lenses from pH 7.6 to 6.8. Although most γ components differed in isoelectric point among these species, a common isoelectric point of one component (pH 7.4–7.5) was found in all. A common gene, M-RNA, and finally protein chain may exist in the lenses of all of these species. The common protein constituents could be an organ specific γ-crystallin.

Supported in part by the Rochester Eye and Human Parts Bank and U. S. P. H. S. grant #EY 0459, of the National Eye Institute.

Intranuclear microtubules during growth and division in Tetrahymena pyriformis *Gl.* WILLIAM D. SULLIVAN, S.J. AND ELINOR M. O'BRIEN.

Intranuclear tubules were observed during growth and division in the macronucleus of the amicronucleate *Tetrahymena.* In exponential cultures, as the lag phase progressed, from one small packet of 6–10 microtubules (2 μ in length; 270 Å in diameter) there was an increase in number and length of tubules and an unfolding at one end demonstrating the beginning of a lateral growth and a unidirectional growth of the tubules. In synchronized cultures during the first burst of division the tubules were observed to be considerably lengthened (3 μ–4 μ), parallel to and concentrated at the macronuclear envelope. At no time were they associated with outpocketings and invaginations of the macronuclear envelope except at division when they penetrated the constriction point. In the divided macronucleus tubules extended into the attached SNA and were observed even after complete separation of the SNA from the daughter nucleus.

The changes noted in the morphology and distribution of the microtubules during growth and division confirm previous theories and elicit new theories regarding the role of microtubules in cells. The unfolding of the microtubules at one end during lag phase is consistent with the current theory of self-assembly of the protein subunits and unidirectional growth. The present investigation proposes that the microtubules act as a transport mechanism operable only during division of the cell and even then not in the conventional sense.

The concomitant growth of the microtubules and disintegration of nucleoli as well as the presence of microtubules in the disintegrating nucleoli indicate strongly that the microtubules have their origin in the nucleoli. This would seem to indicate that the protein subunits of the microtubules are the same protein subunits of which the nucleolus is comprised. Preceding this disintegration of the nucleoli the RNA synthesis has been completed and as the nucleoli disintegrate and the microtubules are formed the RNA is included within the protein subunits.

As the macronucleus prepares for division, the microtubules with RNA extend throughout the length of the nucleus. As the macronucleus divides by constriction the microtubular RNA is deposited in each daughter nucleus and, at the constriction point, into the cytoplasm.

The specific parameters of growth and function, in this particular investigation, therefore, appear as one and the same parameter.

Some factors controlling reproduction in the spider crab, Libinia emarginata. GERTRUDE W. HINSCH.

Observations were made on the hormonal control of molting, sexual maturation and breeding in *Libinia.* Mature ovigerous females can be collected during the summer months of June–September. Immature crabs molt to the mature state during August. These have small, white ovaries containing numerous oocytes which show no signs of vitellogenesis. Vitellogenesis is seen in ovaries of crabs newly molted to maturity or those brooding embryos. Mature females collected during the non-breeding season (winter months) have fully developed ovaries.

The normal breeding season can be extended by ablation of the eyestalks of mature females. Oviposition outside of the breeding season occurs following eyestalk removal. There is no indication of further molting in these females. Eyestalk removal in mature females seems not to alter the breeding cycle or production of pheromone which is attractive to males.

Ablation of eyestalks in immature females leads to precocious molting. Rarely, however, do these females molt to maturity. Following eyestalk removal 50 to 51 crabs retained the immature apron and small ovarian size although carapace length was that of mature females.

The results suggest that in *Libinia,* the eyestalk hormone controls molting. In mature females the eyestalk hormone appears not to inhibit ovarian development but to control the release of oocytes from the ovary.

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AUGUST 18, 1970

On immunologic reactivity to histocompatibility antigens. DARCY B. WILSON.

An unusually large proportion (2–3%) of the lymphocyte population on nonimmunized individuals is capable of being stimulated to proliferate by alien leucocytes of other members of the same species. This mixed lymphocyte interaction (MLI) has an immunologic basis since it reflects the immunogenetic status of the cell donors; it is thought to be the outcome of the recognition of homologous histocompatibility (H) isoantigens by immunologically competent antigen reactive cells (ARCs). However, the large proportion reactive to a single antigen system is an awkward conclusion to accept in view of two constraints of the Clonal Selection Hypothesis of Burnet:—namely, that the range of reactivity of a given ARC is restricted to a single antigenic determinant, and that they occur in low frequency (10^{-5}–10^{-6}) in an unimmunized animal.

Assuming that they are monospecific, there are two general possibilities to account for such a high frequency of ARCs:—(1) that they represent an immunologically amplified clone stemming from prior experience of the cell donors with cross-reactive environmental antigens; and (2) that this particular pool of ARCs is important in some crucial ontogenetic step, that they react to a special class of antigens of limited number, and that their high frequency is brought about by some expansion mechanism under innate genetic control.

Comparison of germ-free-pathogen-free (GFPF) and conventional isogenic Fischer strain rats as cell donors in the MLI has direct bearing on these two possibilities. Whereas lymphocytes from both conventional and BFPF are fully reactive to homologous H isoantigens, lymphocytes of conventional rats display some, but significant amounts of reactivity to heterologous H antigens, and lymphocytes from GFPF rats are totally nonreactive to heterologous antigens. These findings favor the second alternative. Apparently an individual has a higher proportion of ARCs specific for the antigens of other members of his species than those of other species. Detectable reactivity by cells of conventional animals to heterologous species antigens may reflect contact with cross-reactive environmental antigens, but this does not account for reactivity to homologous isoantigens.

An inducible lysin in Limulus polyphemus *with similarities to the complement system of vertebrates: detection, characteristics and dissection from phospholipase A.* HENRY GEWURZ, VANESSA BIRDSEY, DONALD JOHNSON, JEAN LINDORFER, KAY TOWNSEND AND ANITA GEWURZ.

Cobra (*Naja haje*) venom factor (CVF) induces consumption of the six terminal complement (C) components in vertebrate serum and induces "passive lysis" of bystanding erythrocytes via C without requirement for antibody or earlier-acting C components. This passive lysis reaction was used to detect C-like activities in several lower vertebrate and invertebrate sera. We now report investigations of a CVF-inducible lysin in *Limulus polyphemus* with similarities to the C system of vertebrates.

When optimal CVF is added to mixtures of *Limulus* plasma and rabbit erythrocytes (E) in Allen's artificial sea water, marked damage to the E membrane occurs (Step I) which can be visualized as hemolysis and quantified when the cells are resuspended in isotonic saline (Step II). Step I requires high ionic strength (0.63 M NaCl), calcium (9.5 mM), time (60 min) and temperature ($24° > 37° > 4°$ C); Step II occurs rapidly in absence of divalent cations and higher temperatures.

Cell-phase plasma had somewhat greater activity than serum taken post-coagulation, and cell extracts were inactive. *Limulus* plasma retained ability to support lysin formation during 1–5 day storage at 4° C, cycles of freeze-thaw, absorbtions with E and preincubations with 0.04 M hydrazine or CVF itself, while activity was blocked by EDTA or 70° C for ½ hr. The activity (85%) partitioned chiefly with the pseudoglobulins during water dialysis. Preincubation with endotoxic lipopolysaccharides (LPS) led to substantial titer reductions in selected plasmas, while both CVF and LPS regularly induced substantial titer reductions in whole blood.

CVF also induced phospholipase A activity (PAA) in *Limulus* plasma, but PAA seemed dissociable from the CVF-induced hemolysin since it could be generated after preheating plasma to 70° C and partitioned ($> 90\%$) into the euglobulin fraction. PAA generated during the plasma-CVF interaction therefore seems not sufficient (and perhaps not necessary) for inducible-lysin activity.

We conclude that CVF activates a lysin system in *Limulus* plasma with remarkable similarities, and certain contrasts, to the C system of man and anticipate that this lysin will provide a marker to help detect progenitor C and related proteins.

Supported by grants from the USPHS (AI-09806-01), American Heart Association, and the Minnesota Medical Foundation. H. G. is an Established Investigator of the American Heart Association.

Enzyme changes associated with development of bacterial competence. H. T. EPSTEIN.

A working model for the physiological nature of competent cells of *Bacillus subtilis* requires growth in a rich medium to make the cells binucleate, amino acid step-down to stop DNA synthesis and weaken the cell wall, followed by a single cell division step. Incoming DNA is attacked by cell nucleases if suitable restriction signals are not present. Indirect experiments on transfection confirm the drop in DNA polymerase and changes in two nucleases. Direct experiments with sonicates show a 20-fold drop in DNA polymerase activity and an increased activity of a Ca-activated nuclease attacking only single-stranded DNA. Adding Ca to transfection experiments changes phage SP82 transfection in both amount and dependence on DNA concentration; the enzyme is therefore responsible for the nonlinear dependence of SP82 transfection on DNA concentration.

Macrophage activating protein from the sea star Asterias forbesi *coelomocyte simulating effector substances of the delayed hypersensitivity response.* Robert A. Prendergast.

Accumulation of blood borne mononuclear cells in cellular immune reactions is presumed to result from elaboration of chemotactic and macrophage activating substances following the interaction of sensitized lymphocytes with homologous antigen. A protein isolated from the cell sap of the coelomocyte of *Asterias forbesi* fulfills many of the criteria for the biological effects of these mediators whose putative function it is to amplify the cellular immune response by vastly increasing the nonspecific inflammatory cell infiltrate at sites of antigen deposition. Of 32,000 molecular weight, this protein migrates as a gamma 1 globulin and has been purified to the extent that a single arc results on immunoelectrophoresis of one protein peak following G75 Sephadex column chromatography of whole coelomocyte cell sap. Intracutaneous injection of as little as 1 microgram of this protein in normal guinea pigs, sheep, mice, rats and rhesus monkeys results in a lesion which is physically, temporally and histologically indistinguishable from the classic delayed hypersensitivity reaction of the tuberculin test in animals previously sensitized to that antigen. Purified peritoneal macrophages free of lymphocyte contamination are inhibited from migrating out of capillary tubes in the presence of the star protein indicating that cross reactivity with some ubiquitous antigen is unlikely as a cause of the skin reactivity seen in all animals thus far tested. Macrophages exposed to this factor either *in vivo* or *in vitro* demonstrate an increased content of lysosomes and are capable of inflicting severe damage on underlying L cell or fetal mouse fibroblast monolayer cultures. The star protein itself is not toxic to these indicator cells in the concentration employed (5 micrograms/ml medium). These data suggest that this material acts both as a chemotactic and cell activating factor specifically on the macrophage population of a number of mammalian species, and further that there exists a distinct analogy between the star coelomocyte protein and the mediators of cellular immune reactions in the vertebrates.

GENERAL SCIENTIFIC MEETINGS

August 24–27, 1970

Abstracts in this section are arranged alphabetically by author. Author and subject references will be found also in the regular volume index, appearing in the December issue.

T. H. Morgan, Richard Goldschmidt and the opposition to Mendelian theory 1900–1940: a chapter in the history of biological ideas. Garland E. Allen.

It is generally thought that the Mendelian theory was well received after the rediscovery of Mendel's paper in 1900. In reality, the theory faced serious criticism, especially between 1900 and 1915, but even through the 1930's and 1940's. One of the strongest critics before 1910 was T. H. Morgan (1866–1945) whose own work with *Drosophila*, starting about 1909, convinced him of the value of Mendel's scheme (studies of Morgan's criticisms have been published previously). Another equally strong and more persistent critic was Richard Goldschmidt (1878–1958), long director of the Kaiser Wilhelm Institute and after 1936 Professor of Zoology at Berkeley. Trained as a descriptive morphologist, Goldschmidt became interested in genetics through his studies of sex determination and the evolution of melanism in the nun and gypsy moths. His physiological theory of sex determination (he saw sex as a balanced state) led Goldschmidt to view heredity as a *process*. He opposed the Mendelian-chromosome theory of the Morgan school because it emphasized genes as discrete, inviolable units or structures and did not account for their function in chemical or physical terms. To Goldschmidt, Morgan had developed primarily a morphological theory of heredity. While Morgan was aware of the necessity of some day trying to study the physiology of genes, he felt that in the years 1915–1930 experimental methods were not available for investigating this problem. Goldschmidt was particularly incensed at "explanations" such as position effect, advanced by Sturtevant in 1925, which seemed to be only pure speculation designed to save the Mendelian hypothesis. Goldschmidt's own concept of the chromosome as a "continuum"—a single long molecule with no discrete genes—was hardly an improvement. But his persistent attacks on the structural theory of Morgan kept alive the very real problem of how genes function in a physiological sense.

The effects of cytochalasin B on cytoplasmic movement, cleavage, and subsequent development of the squid embryo, Loligo pealei. John M. Arnold and Lois D. Williams-Arnold.

Earlier investigations have demonstrated the existence of a morphogenetic pattern of developmental information in the egg cortex which is transferred to the yolk epithelium (= periblast) prior to organogenesis (Arnold, 1965, 1968). To check the role of cytoplasmic movements in the establishment of this morphogenetic pattern, naturally fertilized embryos were pulsed at various times for ten minutes with 0.2 μg/ml of cytochalasin B in dimethylsulfoxide dissolved in sea water. After the pulse, the embryos were returned to normal sea water and development followed. Higher concentrations (2.0 μg/ml) or longer treatments stopped cytoplasmic streaming in precleavage eggs and blobs of cytoplasm appeared on the surface of the embryos. When embryos were pulsed between first and second meiosis the blastoderm appeared normally but the cleavage pattern became radial rather than bilateral. Cellulation and subsequent development was normal until organogenesis when 40% of the embryos developed abnormalities ranging from a complete lack of recognizable organs to reduced and distorted organs in their usual positions. When pulsed during first mitosis, first furrow formation was prevented or delayed. 78% of the resultant embryos showed abnormalities and of these many more lacked recognizable organs or had organs in abnormal positions. Pulsing at four cells killed many embryos but those that survived had reduced recognizable organs in their proper placement. Pulsing at 32 cells produced 78% abnormal embryos all of which had reduced recognizable organs in their proper placement. Pulsing when the embryo was one-third or three-fifths cellulated produced no significant defects.

It was concluded that cytochalasin B sensitive cytoplasmic movements are important in establishing the blastodisc and positioning the mitotic apparatuses which in turn determine the cleavage pattern. Abnormal cytoplasmic movements lead to an abnormal yolk epithelium which then causes an anomalous pattern of induction resulting in a failure of organogenesis or incompletely differentiated embryos. Thus, the precleavage pattern of cytoplasmic movement seems to determine the organization and position of the organ determining areas in the egg cortex and greatly influence subsequent differentiation of the squid embryo.

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Effect of fertilization on the calcium and magnesium content of the eggs of Arbacia punctulata. Roobik Azarnia and Edward L. Chambers.

Jelly-free unfertilized eggs were prepared in 0.2% suspension in sea water, divided between two beakers, stirred at 60 rpm, maintained at 20° C, and one of the suspensions inseminated. At intervals duplicate 100 ml samples were removed to Hopkin's tubes, hand centrifuged at 250 × *g* for 30 sec, the supernatant aspirated, and the volume of eggs plus supernatant fluid in the calibrated tails of the Hopkin's tubes measured using a cathetometer.

Unfertilized eggs in sea water contain on the average 2.98 ± 0.04 μm Ca/ml eggs and 21.9 ± 0.3 μm Mg/ml eggs. Stirring for periods of 11 to 239 minutes, a three minute wash with artificial sea water containing 1/10th the normal concentrations of Ca and Mg, or with Ca-free sea water, and treatment with 0.1% pronase to remove the external coats had no effect on the Ca and Mg content of unfertilized eggs.

Following fertilization a sharp increase in the Ca content (15% at 3 min) occurs followed by a sharp decrease with the Ca content attaining the unfertilized level by 40 min after insemination. In parallel determinations carried out on eggs washed 3 min in Ca-free, or 1/10th calcium sea water a 20 to 40% decrease in the Ca content of each of the egg samples was observed compared to the corresponding samples in sea water. The result of this decrease is that the washed eggs exhibited a sharp (15%) lowering of the Ca content at 6 min after fertilization attaining a value 25% less than that of the unfertilized eggs by 40 to 50 minutes after insemination.

With regard to the Mg content, a 3% (but statistically insignificant) increase is observed by 4 minutes after insemination. Thereafter a sharp decrease occurs until by 50 minutes the Mg content is 25% less than that of the unfertilized eggs. Washing the fertilized eggs in low Ca and Mg sea water had no effect on the Mg content.

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A virus disease of the shore crab Carcinus maenas. FREDERIK B. BANG.

When blood is taken from a shore crab by a syringe and needle in the presence of an extract of amoebocytes, a clot starts to form in a few seconds, and within a few minutes this contracts on the slide into a tight mass. In the summer of 1969, while at the Station Biologique, Roscoff, a crab was found in which typical clotting did not occur. When this non-clotting blood was injected into other crabs, the same disease process was induced 2 to 13 days later. The infection was repeatedly transferred and was manifested by (1) failure of clotting, (2) reduction in total amoebocyte count, (3) formation of small tight clumps of amoebocytes in the peripheral tissue. The agent was filter passing, and was preserved by freezing of the blood.

Four aspects of this infection have been studied at Woods Hole. (1) Mortality, at least during the first month, is relatively mild and recovery as manifest by a reappearance of the ability to form a firm circular clot is high. Recovered animals are susceptible to reinoculation. (2) The agent tested by inoculation of other crabs persists in the blood as long as seven weeks after inoculation and several weeks after the blood has regained the ability to clot. (3) The agent in serum titered to 10^9. (4) Electron microscopy of infected amoebocytes fixed two days after initiation of signs of infection showed typical virus particles, roughly 100–150 mμ which occurred in packages within the cytoplasm, in scattered arrays within the endoplasmic reticulum, and on the surface of the cells. No particles were found in normal amoebocytes with or without clot formation.

Fine structure and intracellular responses of photoreceptors of a pelagic tunicate, Salpa. STEPHEN N. BARNES, ANTHONY L. F. GORMAN AND JOHN S. MCREYNOLDS.

Visual cells in chordates, including vertebrates, have been shown to be of the ciliary type. Recent evidence indicates that ciliary type photoreceptors are associated with hyperpolarizing receptor potentials. The urochordate *Salpa,* has a lensless eye, arising from the neural ganglion, which is directly accessible to light, being situated on the dorsal surface of the animal beneath the transparent tunic. Sections of the eye examined with an electron microscope show that the visual cells are approximately 10 micra in cross-section and 60 micra in length, and give rise to a randomly organized array of microvilli at one end as their photosensitive structure. No membrane specializations were seen which were derived from cilia. Intracellular recordings were made from these cells with high resistance, KCl-filled microelectrodes. Resting potentials in the dark were low, approximately 10 mv inside negative. The response to illumination was a hyperpolarizing potential change, graded with light intensity, which could be up to 70 mv in amplitude at maximum intensities. The responses were sustained as long as the light was on. No spike activity was seen in these cells. Changes in the voltage drop produced by constant current pulses passed across the cell membrane in darkness and during illumination showed that the hyperpolarizing receptor potential is associated with an increase in membrane conductance.

This investigation was supported in part by Training Grant number GM01981 and Research Grant number EY00443 from the National Institutes of Health.

Comparison of melanophorotropic lipoprotein fractions from the pituitary of the dogfish, Mustelus canus, *and from the eyestalk of the fiddler crab,* Uca pugilator.

CLELMER K. BARTELL, MILTON FINGERMAN AND JOHN E. STEPHENSON.

Ethanol and 95% methanol:chloroform (2:1) extracts of *Uca* eyestalks contain lipoprotein components which, in aqueous media, form low-density micellar lipoproteins that cause dispersion of pigment in *Uca* melanophores. At concentrations above one eyestalk per animal the activity of both extracts is higher than that of aqueous extracts. The melanophorotropic factor is more stable in methanol:chloroform than in ethanol and will survive filtration on Sephadex LH-20 (methanol:chloroform) with little loss of activity. Extracting in methanol:chloroform both optic ganglia and sinus grands yield melanophorotropic lipoprotein. The density for the active micellar lipoprotein from ethanol extracts is between 1.016 and 1.034 g per ml, for methanol:chloroform, between 1.034 and 1.047 g per ml. When the active material is extracted in methanol:chloroform from lyophilized, chloroform-washed tissue and separated on LH-20 (methanol:chloroform), the micellar lipoprotein density is not changed. A higher density active material which appears to be a pseudomolecular lipoprotein, or apolipoprotein, with a density between 1.06 and 1.20 g per ml has been separated using two diphasic solvent systems—methanol:chloroform:water (2:1:1) for methanol:chloroform extracts and ethanol:chloroform:water (2:1:1) for ethanol extracts. In each system the melanophorotropic material was recovered in the upper phase, *i.e.,* the methanol:water, or ethanol:water phases. The apolipoprotein from both extracts, when suspended in aqueous media with lipid components from either extract, will associate with micelles having the same density characteristics of the lipoprotein from the whole extract of its origin. The apolipoprotein, therefore, appears to show some specificity. A lipoprotein (density between 1.016 and 1.064 g per ml) that will cause dispersion of pigment in melanophores of dogfish was extracted from *Mustelus* pituitary using 95% methanol:chloroform (2:1). The presence of melanophorotropic lipoproteins in two animals of diverse phylogenetic origins is evidence that the association between lipids and peptide hormones in cells of endocrine tissue may be a general phenomenon.

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Movements of echinochrome granules during the early development of Arbacia *eggs.*

ANN M. BELANGER AND RONALD C. RUSTAD.

The echinochrome pigment granules in *Arbacia* eggs are known to exhibit random saltatory movements before fertilization and migration to the cortex shortly after fertilization. Before the sixteen-cell stage nearly all of the granules leave the region which will become the micromeres. In our experiments this migration occurred during the four-cell stage, leaving a "clear area" on each blastomere.

Incubation at 5° C reversibly inhibited migration to the cortex and formation of "clear areas." The pigment granule movements occurred at the normal times following γ-irradiation of unfertilized or fertilized eggs with doses as large as 250 kR. Colcemid ($10^{-3}M$) did not prevent prefertilization saltation, migration to the cortex, or formation of "clear areas." However, the latter was often delayed. Cytochalasin B (1–5 μg/ml) reversibly blocked saltation and cortical migration and prevented the formation of "clear areas." Cytochalasin activated the elevation of the fertilization membrane in unfertilized eggs. When activated eggs were washed, pigment granules migrated to the cortex, and "clear areas" were formed at the normal time. When cytochalasin was added shortly before cytokinesis, division furrows began to form and then regressed, leaving concentrated bands of pigment in the furrow regions.

Since blocking mitosis in prophase with irradiation or in metaphase with Colcemid did not prevent the formation of "clear areas," this phenomenon must be independent of nuclear activity. Since the normal movements of pigment granules occur in the presence of Colcemid, but not cytochalasin, these movements would appear to be independent of microtubules and dependent on functional microfilaments.

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The statistical phenotype concept, with applications to skeletal variation in the mouse. ALLAN BIRNBAUM.

Genetic theory gains conceptual unity, simplicity, and generality with adoption of the concept of a *statistical phenotype,* which includes as special cases the two kinds of variation usually considered (qualitative and continuous), and also threshold characters, general multinomial classifications, and any others. A statistical phenotype is defined formally simply as a probability distribution over any given set of phenotypes (descriptions). This leads to some new systematic approaches to genetic data analysis, including Mendelian and related interpretations, freed of the usual conceptual restrictions and practical difficulties associated with questions of scaling. It sometimes also provides a useful standpoint for appraising the reality and nature of a possible underlying scale of variation.

The general approach is introduced briefly here; it has been developed independently and more systematically by Collins (*Genetics,* 1967, 1968, 1969, 1970). It is applied here in detail to the data on skeletal variation in the mouse of Green (*Genetics,* 1954, 1962), and the results are compared and contrasted with those found by Green by use of the more customary methods. Many of Green's interpretations concerning the adequacy of one-gene and several-gene models are given new support; for other interpretations, some qualifications or modifications are indicated.

Studies on blood folic acid in the smooth dogfish, Mustelus canis. T. A. BORGESE AND L. CIANCI.

Heparinized caudal vein blood, obtained from a single dogfish or pooled from several, was used for determining blood folic acid employing the microbiological assay with *Lactobacillus casei* as the test organism. Most of the folic acid present in the blood is found in the cells. The mean values and standard errors for whole blood, plasma, and cells from ten experiments were 19.4 ± 4.6, 2.32 ± 0.48, and 72.1 ± 22.0 ng per ml, respectively. Several factors, including suspension medium, plasma concentration, and *in vivo* maturation of red cells, were shown to influence folic acid levels. Cells washed and resuspended in buffered elasmobranch Ringer's solution, pH 7.2, showed considerably less measurable folate activity than cells washed and resuspended in their own plasma. In each case the cells were reconstituted to the same packed cell volume. The ability of plasma to maintain maximum folate activity in unwashed cells decreased as increasing volumes of plasma were replaced with Ringer's.

After removal of the buffy coat, differential centrifugation separated the red cells into relatively young (less dense) and relatively old (more dense) population. Bioassays on each of these demonstrated that the less dense cells had a higher folate content than the more dense ones.

We conclude that a factor, present in the plasma, is essential for maximum folate activity in the dogfish red cells.

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Microspectrophotometry of visual pigment and spectral sensitivity of retinular cells in the crab, Carcinus. M. S. BRUNO, M. I. MOTE AND T. H. GOLDSMITH.

Single rhabdoms from dark-adapted green crabs (*Carcinus maenas*) were detached from their surrounding retinular cells and suspended in 5% glutaraldehyde. Transmission spectra were recorded using a 4 × 10 μm measuring beam incident at right angles to the long axis of the rhabdom. Difference spectra obtained by partial bleaches with red followed by yellow light were almost identical and gave no indication that more than one visual pigment was present. The mean difference spectrum for the total bleach of 23 rhabdoms had λ_{max} at 502–505 nm and had 0.6% μm^{-1} absorption at the peak.

Spectral sensitivities (reciprocal photons for a 5 mv response) of single retinular cells were measured at 9 wavelengths between 412 and 672 nm in preparations of isolated eyestalks, using intracelluar microelectrodes. Average sensitivity of six cells was found at 495 nm, in agreement with the results on 17 other cells which were held only long enough to measure spectral efficiency (response height for an equal photon spectrum). The spectral sensitivity of the ERG was similar, except that the curve was slightly broader at longer wavelengths, presumably due to the relative transparency of the interommatidial screening pigment in the red. Selective adaptation of single cells with either red or blue light decreased the sensitivity but rarely changed the shape of the curve from that of the dark-adapted cell. In a few cells, red adaptation caused a decrease in relative sensitivity at the red end of the spectrum. This decrease was associated with slowly occurring changes in sensitivity which made accurate measurements difficult. The time course of these changes suggest that they may have been due to retinomotor phenomena.

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Pacemaking and phrase-shifting in synchronizing fireflies. JOHN BUCK, JAMES F. CASE AND FRANK E. HANSON.

In certain oriental firefly species in which the males habitually flash rhythmically in unison it was found possible to entrain individuals to artificial light flashed in both slower and faster rhythms. In this entrainment the firefly systematically led when paced at a rhythm slower than his spontaneous rhythm and lagged when paced at a faster rhythm. The amount of lead or lag approximated the difference between the pacer period and the firefly's free-running flash period. The firefly's interflash period immediately after the first pacer flash was typically longer or shorter than the pacer period depending on whether or not the flash that would next have occurred, had the firefly persisted in his original rhythm, was inhibited. The phase-shifting effect of the pacer flash was immediate but lasted only one flash cycle—that is, the firefly reverted to his original free-run flashing frequency as soon as the pacer ceased flashing. Such entrainment by artificial light could be explained if each pacer flash in effect resets the firefly's endogenous central nervous pacemaker.

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Comparative physiology of neurally controlled luminescences. J. F. CASE, G. T. REYNOLDS, J. BUCK, J. BURNS AND R. HALVERSON.

The mechanisms of neural control of luminescence have almost totally evaded analysis except in firefly larvae and adults in which adrenergic control seems quite likely. The study of three other systems now suggests that they also are adrenergic.

Railroad worm, *Phryxothrix,* thoracic and abdominal photophores are neurally excitable and glow in an erratically phasic manner when attached to an eserinized ganglion. Pseudoflashes are induced by re-oxygenation of hypoxic preparations. Isolated photophores glow in nor-adrenaline and amphetamine, but not in acetylcholine. Nor-adrenaline restores glowing in amphetamine exhausted photophores. Midshipman fish, *Porichthys,* photophores are excitable *via* their peripheral innervation *in vivo* and *in vitro* and glow when treated with nor-adrenaline or amphetamine. Acetylcholine and eserine *in vivo* together induce weak glowing but only after elapse of sufficient time to render a direct effect improbable. The sea pansy, *Renilla,* glows in a sustained fashion in amphetamine or ephedrine but undergoes no luminescence change in nor-adrenaline. Luminescence is inhibited by glutamate without evidence of initial enhancement. Thus all probably directly neurally excitable luminescent systems studied to date in some reasonable detail appear to have similar transmitter mechanisms.

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The effect of temperature on the efflux of ^{45}*Ca from the eggs of* Arbacia punctulata. EDWARD L. CHAMBERS, ROOBIK AZARNIA AND WILLIAM E. MCGOWAN.

Jelly-free unfertilized eggs were prelabelled in a 1.2% suspension containing 2 μc ^{45}Ca/ml, 1 mg streptomycin/ml, pH 8.0, 20° C, stirred at 30 rpm for 12 hours. The eggs were washed free of external ^{45}Ca by repeated addition and decantation of inactive sea water over a two hour period, and then inseminated.

Efflux from the washed unfertilized eggs occurs according to a single term exponential process with a rate constant of 0.0003 min^{-1} (initial efflux for the first several minutes after removal of the unfertilized eggs from labelled sea water was omitted, since this is exceedingly variable and represents desorption from the external coats of the eggs).

Following insemination, efflux occurs according to a double exponential process, with an average rate constant at 20° C for the fast component of 0.074 min^{-1} (2.5×10^{-12} moles Ca cm^{-2} sec^{-1}) and 0.0026 min^{-1} (9×10^{-14} moles Ca cm^{-2} sec^{-1}) for the slow component. At 10° C the rate constants for the fast and slow components are decreased to 0.030 min^{-1} and 0.00077 min^{-1}, respectively. This gives Q_{10} values for the rate of ^{45}Ca efflux from the fast component of 2.5, and 3.4 for the slow component, indicating that both phases are linked to the metabolic activity of the egg.

Further evidence that the measured efflux of ^{45}Ca is intracellular in origin is (1) exposure of washed prelabelled unfertilized eggs to 0.1% pronase and to 0.1 to 0.5 m*M* lanthanum in bicarbonate and sulfate free sea water do not significantly diminish the ^{45}Ca content, (2) the same double exponential efflux curves were obtained following fertilization whether the eggs had been prelabelled for 50 minutes or for 24 hours, and (3) the decrease in Ca and ^{45}Ca content of fertilized eggs washed in Ca-free sea water, when expressed as per cent of the unfertilized values, are the same.

Supported by NSF grant GB 8054.

Failure of coelomocytes of some Asterias forbesi *to clump on glass.* JOHN NORRIS CHILDS III.

Clumping of coelomocytes of the starfish *Asterias forbesi* involves the formation of loose aggregates of cells and subsequent contraction over three to five minutes to form tight clumps. Clumping was studied in the star since it has a single cell type in a coelomic fluid containing only small amounts of protein. Previous work has shown that a crude extract of coelomocytes clumps these cells *in vivo* and in an *in vitro* buffer system of pH less than 5.8 which prevents cells from spontaneously aggregating. Of 300 starfish examined, 31 had cells which failed to clump on glass. These coelomocytes appeared morphologically similar to normal cells and responded normally to the addition of coelomocyte extract *in vivo* and *in vitro*. *In vitro* tests of clump initiating ability of various substances showed this ability to be present in coelomocyte extract and protein isolated from coelomic fluid, but lacking in ATP, ADP and sperm and ova extracts.

There was a correlation between presence of sperm in coelomic fluid and failure to clump. Two early findings showed a ciliated parasitic organism, *Orchitophyra stellarum,* to be present with free sperm in the coelomic fluid of non-clumpers. Upon injecting non-clumpers with coelomocyte extract, sperm seething with ciliates was discharged, establishing a correlation between maleness, infection of testes with *Orchitophyra stellarum* and failure to clump.

In spite of failure to clump spontaneously on glass, the coelomocytes of these non-clumpers did clump in response to the two substances, coelomocyte extract and coelomic fluid protein. A difference observed in the time course of response and final result may indicate a clumping process of several steps, involving a plasma factor initiating loose aggregation and subsequent secretion of a cellular substance promoting tight clumping.

Changes in fluorescence of squid axons during activity. L. B. COHEN, D. LANDOWNE, B. B. SHRIVASTAV AND J. M. RITCHIE.

In an attempt to study changes in membrane structure associated with nerve activity three dyes were applied to the squid giant axon and their fluorescence was monitored during voltage clamp experiments. Signal averaged records of Acridine Orange fluorescence showed a decrease in fluorescence by about 3×10^{-5} when the nerve was hyperpolarized by 50 mv and an increase of the same amount when the nerve was depolarized by 50 mv. Rhodamine B exhibits a similar behavior except that the sign of the change is reversed. As the experiment progresses the amplitude of the change in Rhodamine B fluorescence decreases and then the change turns over and is in the opposite direction. The early change is an increase of about 10^{-5} for a 50 mv hyperpolarization and the late change is a decrease of approximately 2×10^{-5} for the same step. With both Acridine Orange and Rhodamine B the same results are obtained if the dye is micro-injected inside the axon or applied in the outside sea water.

1-analino-8-napthalene sulfonate (ANS) shows opposite changes when applied to opposite sides of the membrane. Thus when ANS is microinjected a 50 mv hyperpolarization causes an increase in fluorescence of about 3×10^{-5}. When the dye is applied externally this voltage step produces a decrease of the same size. In addition, in records from microinjected axons, one can see a difference between hyperpolarization and depolarization, hyperpolarization being about 30% less effective in causing the change in ANS fluorescence.

The time course of the fluorescence changes follows the voltage with a lag of less than one msec (different lags for different dyes). The changes in fluorescence in these dyes thus seems to be related to the changes in voltage across the axon membrane.

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The possible role of hydrogen cyanide in the origins of life: the hydrogen cyanide polymers. D. B. Donner, A. P. Lobo, J. D. Wos and J. P. Ferris.

The chemistry of hydrogen cyanide has been studied with respect to its role in the origins of life. Hydrogen cyanide couples in aqueous media (pH 8–9) to yield a tetrameric species which serves as an intermediate in the formation of most of the purines of importance in contemporary biological systems.

Hydrogen cyanide also self condenses to yield polymers; these reactions occurring under the same conditions that lead to purine synthesis. Polymers containing a repeating subunit (with attendent functionality that imparts to these compounds acidic, neutral, or basic character) have been isolated and separated from one another using Sephadex and ion exchange chromatography. A molecular weight of five hundred is estimated from Sephadex data and sedimentation studies. The structures of the polymers formed are independent of the base used to start the polymerization suggesting that the reaction is initiated by attack of cyanide ion on hydrogen cyanide tetramer.

Hydrolysis in six normal hydrochloric acid degrades twenty to twenty five per cent of the polymers to at least eleven amino acids. Neither the amount of amino acids liberated nor the character of the non-degraded portion of the polymers varies significantly when the base used to neutralize the hydrogen cyanide is ammonia, sodium hydroxide, or triethylamine.

The possibility of peptide bonds being present in the polymers has been disproved. Such functionality is inconsistant with some of the physical properties displayed by the polymers. Degradation experiments using pronase and carboxypeptidase A (both of which attack peptide bonds) failed to liberate amino acids. The Biuret test was also negative indicating the absence of peptide bonds.

A new structure is proposed for the polymers consisting of a repeating ring skeleton (which is non-degradable in acid) with labile side chains that are hydrolized to amino acids. The polymers catalize the oxidation of NADH to NAD and resorcinol to *o*-benzoquinone. Further studies on the structural and catalytic properties of the polymers are in progress.

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S-potentials in the skate retina: intracellular responses during light- and dark-adaptation. J. E. Dowling and H. Ripps.

The S-potentials recorded from the all-rod retina of the skate probably originate in the large horizontal cells located immediately below the layer of receptors. These cells were always hyperpolarized by light of any effective wavelength. The adaptive properties of S-units were investigated by measuring changes in sensitivity and D. C. level during light- and dark-adaption. After flash bleaching the retina, the variation in log threshold followed the same time course as observed previously for the ERG b-wave and ganglion cell discharge. The effect of the flash on the D.C. level was to maximally hyperpolarize the S-unit for about 10 min during which time no response could be elicited by test flashes 8 log units above absolute threshold; with further time in darkness the D.C. level moved back toward the cell's resting potential and excitability returned. Subsequently changes in sensitivity exhibited an initial fast (neural) phase and a later slow (photochemical) component, but neither appeared to be correlated with the temporal course of the changes in D.C. level.

The increment threshold function measured against steady background fields was also similar to that obtained from more proximal regions of the retina. When the background was sufficiently bright to maximally polarize the S-unit, the cell became unresponsive for periods up to 15 min (depending upon background intensity). However, the cell did not maintain its D.C. level with continuous illumination; as the cell's potential returned toward the resting level, response amplitude increased, and thresholds decreased to a steady state value.

The waveform and rise and fall times of the S-potentials were markedly different in the light- and dark-adapted states. However, amplitude *versus* intensity functions determined under light- and dark-adapted conditions for wavelengths from 420 to 620 nm were similar, and the spectral sensitivity curves derived from these data were virtually identical.

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Differential utilization of phytoplankton food resources by marine ectoprocts.

JUDITH W. DUDLEY.

The general view of filter-feeding by ectoprocts is that they are remarkably alike in the food gathering apparatus, with probable identity in their food resources. Several lines of evidence suggest instead that individual species of ectoprocts found near Woods Hole are specialized for making use of the available food resources.

In these species tentacle number varies from 8 to 30, tentacle length from 0.2 to 1.0 mm, mouth diameter from 0.02 to 0.09 mm, and the diameter of the food gathering cone (maximum distance across the expanded lophophore) from 0.2 to 1.4 mm. All of these variables are positively correlated, with the greatest number and longest tentacles occurring in species with the largest mouths and food gathering cones. These species would therefore be able to utilize different size fractions of the phytoplankton.

Experimental feeding of micronic beads to two species of *Bugula* with similar sized zooids showed that though they were consuming beads from within the same size range, the species with the larger mouth was able to consume more large beads, and the species with the faster digestion time was able to consume significantly more beads per zooid.

In view of these observations, it appears that a better initial premise for considering feeding by ectoprocts from a given locality is that every species is doing something different from every other. This may be important in considering the diversity of marine ectoprocts, with more than 3000 living species.

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Water permeability of Fundulus *eggs.*

P. B. DUNHAM, A. CASS, J. P. TRINKAUS AND M. V. L. BENNETT.

Fundulus eggs develop normally in both distilled water and sea water. There is no morphological evidence for a water regulating mechanism which might be responsible for maintenance of constant volume. Accordingly, we determined tracer water permeability, P_D, of fertilized *Fundulus* eggs from H^3-water fluxes at 23° C. For influxes, mid-gastrula eggs from one female were placed in 2× Holtfreter's medium with tracer. Eggs were removed at intervals, washed in unlabelled medium, and extracted in Bray's solution, which was then counted. Volumes and surface areas were calculated from linear dimensions of the egg bounded by the plasma membrane, assuming the shape to be an oblate spheroid. The saturation level of H^3-water, necessary for calculation of k, the rate coefficient of influx, was calculated from the volume of the egg, assuming 80% water space. With this assumption, a semilogarithmic plot of the influx data fit well with a straight line. In three experiments, k ranged from 0.15 to 0.17/

hour. P_D, given by the product of k and the volume/surface ratio, ranged from 0.96 to 1.14 × 10^{-6} cm/sec.

Efflux of H^3-water was measured by transferring labelled eggs through aliquots of unlabelled medium. The k for efflux was similar to that for influx (0.12/hour after 18 hours of loading, 85% exchange of egg water content), although there was an indication of series compartments, since efflux was faster after 1.5 hours of loading (0.17/hour, 20% exchange). The rate of efflux was identical from intact and dechorionated eggs, showing that the plasma membrane is the limiting barrier for water permeation.

The low P_D indicates no special mechanism is necessary for regulation of volume. The observed rate of volume change during development of teleost eggs is consistent with the P_D. The P_D of *Fundulus* eggs, 10^{-6} cm/sec, is one of the lowest reported for thin membranes (less than 100 A). Salmon eggs have recently been shown to have a similar P_D at 5.5° C. The lowest permeability of artificial lipid bilayers is about 10^{-4} cm/sec.

The effect of cold exposure on protein synthesis in the hepatopancreas of the spider crab, Libinia emarginata. REBECCA ELLISON.

The spider crab, *Libinia emarginata,* is found from Nova Scotia to the Bay of Mexico. As a species it encounters a wide range of temperatures. The question asked in these experiments was whether individuals of this species could be stimulated by cold exposure to undergo compensatory adjustment of metabolism like that reported for certain other poikilotherms.

Male intermolt *Libinia* of medium size (approx. 150 g) were used. Experimental animals were kept at 10–14° C in running sea water aquaria for 1, 5, 12, and 18 days. Controls were kept at 22° C. Crabs were starved for an appropriate period to match nutritional status of control and experimental animals. Experiments were performed at the same time of day to minimize the effect of circadian rhythms.

Crabs were injected intrapericardially and protein synthesis in the hepatopancreas (a major site of metabolic activity in the non-molting crab) was measured by the incorporation into protein of a mixture of fifteen C^{14} amino acids (5 μc/crab) after a 30 minute incubation period at 23° C. The ratio of incorporation into protein to free radioactivity in the tissue was taken as the index of protein synthetic capacity. The group of 14 control animals showed a protein synthetic index of 0.28 ± 0.08. Animals kept at 10° C for 1, 5, and 12 days ($N = 25$) did not show significant differences in mean value from the controls, however individual variations were unusually great (standard deviations about 0.17). One group ($N = 10$, 18 days at 10° C) had a synthetic index of 0.15 ± 0.07 at 20° C indicating loss of metabolic capacity under these experimental conditions. In general, the results indicate no compensatory temperature acclimation in this species, however further studies under other conditions of feeding and handling are needed.

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In vivo *intestinal absorption of D-glucose in the toadfish,* Opsanus tau, *and the rainbow trout,* Salmo gairdneri. A. FARMANFARMAIAN AND F. E. DIBENEDETTO.

The uphill transport of sugars and amino acids in the intestine of mammals is Na-dependent. It is useful to investigate the intestinal absorption of nutrients in marine and fresh water fish since the intestinal lumen of these fish may be exposed to high or low sodium concentrations.

In vitro preparations of toadfish everted sacs undergo contraction after excision and are not suitable for absorption studies. Therefore, *in vivo* procedures were adopted. Fish were weighed and anesthetized in appropriate solutions of MS-222. The midgut was exposed without damage to the circulation. A 3–5 cm segment of the intestine beyond the entry of the bile duct was washed with saline and isolated by means of an anterior and posterior ligature. Solutions of glucose and inulin in the appropriate saline were placed in the segment, mixed, and an initial sample was removed. The abdominal incision was closed properly. The fish were then allowed to revive and swim in a suitable tank or were provided with a flow of oxygenated anesthetic solution over their gills. The absorption was allowed to proceed for 30 minutes at 20 ± 1° C. The body cavity was reopened and a terminal sample of the luminal medium and the entire isolated gut segment were removed for analyses. When an initial solution of 10 mM glucose was used, the anesthetized toadfish gave an absorption rate of 6.83 ± 0.81 μmoles/g fresh gut/hr. The rate for unanesthetized animals (6.86 ± 1.17) was not significantly different ($P < 0.05$). The tissue/medium (T/M) terminal concentration ratio for the former was 0.80 and for the latter was 0.33. The difference was significant and probably due to better circulation in the swimming fish. These ratios did not indicate uphill absorption of glucose. However, at 2 mM initial glucose, the absorption rate dropped to 3.47 ± 0.61 and the T/M was 1.58 indicating that uphill transport of glucose could be observed under those conditions. In trout, at 10 mM initial glucose, a higher absorption rate, 8.89 ± 3.2, was found, and T/M was 0.37.

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Analysis of the chromatophorotropic activity of the central nervous system of the horseshoe crab Limulus polyphemus. MILTON FINGERMAN, CLELMER K. BARTELL AND ROBERT A. KRASNOW.

Extracts of the central nervous system (CNS) of the horseshoe crab were known to evoke pigment dispersion in the melanophores of the fiddler crab, *Uca,* and concentration of the pigment in its leucophores. The present experiments were designed to compare the chromatophorotropic material from *Limulus* with that in the eyestalks of the fiddler crab. Exracts of *Limulus* CNS causd melanin dispersion and white pigment concentration in the fiddler crab, *Uca pugilator,* but were not effective in either dispersing or concentrating the pigment in the crab's erythrophores. In contrast, the eyestalks of the fiddler crab are known to contain, in addition to melanin-dispersing and white pigment-concentrating substances, hormones that evoke both red pigment-dispersing and pigment-concentrating responses. The pigment-dispersing activities from *Uca* eyestalks were eluted from a Bio-Gel P-6 column with an R_f of 0.6 and the pigment-concentrating activities with an R_f of 0.28. In contrast, the melanin-dispersing and white pigment-concentrating activities from *Limulus* CNS had relative flow rates of 0.57, which is essentially the same value as that of the pigment-dispersing activities from *Uca* eyestalks. Furthermore, the activities from *Limulus* CNS did not separate from each other on a column of Sephadex LH-20 in methanol:chloroform (2:1). When *Limulus* CNS was first extracted in ethanol or methanol:chloroform (2:1) rather than directly in saline, both activities were more than three times as large at a concentration of 0.1 equivalent CNS per dose as the corresponding activities obtained with extracts prepared directly in saline. Both activities from *Limulus* CNS were insoluble in acetone and may be due to a single substance.

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The origin of the light producing cells during development of the Ctenophore Mnemiopsis leidyi. GARY FREEMAN AND GEO. T. REYNOLDS.

The origin of the cells which produce luminous products in the radial canals of *Mnemiopsis* has been studied by removing blastomeres from embryos at early cleavage stages of development and monitoring the ability of the larval fragments that form to produce light with a photomultiplier and an image intensifier.

The division which forms the eight cell stage produces the first two classes of blastomeres which develop in different ways—the E and M macromeres. The M macromeres are the only class of blastomeres isolated at this stage which develop the ability to produce light. During the next two divisions (16 and 32 cell stages) the M macromeres give off two micromeres which form the contribution of these macromeres to the ectoblast of the embryo. Light production does not occur if the M macromeres are removed at these two stages indicating that the capacity to produce light is not associated with the micromeres formed at these stages.

In a 24 hour old larvae there are four luminous areas which correspond to the radial canals of the four sets of comb plates. The relationship between the M macromeres and bioluminescence was studied by removing one or more macromeres at the 32 cell stage of development and counting the number of luminous areas which form. The maximum number of luminous areas present is correlated with the number of M macromeres which are present at the 32 cell stage.

Single M macromeres isolated from the 8, 16 and 32 cell stage animals will produce light. However the percentage of cases which produce light declines when the macromeres are isolated from the 16 and 32 cell stages. The relative amount of light produced also declines roughly 500–1000 fold. By increasing the amount of tissue isolated, by culturing two or four M macromeres as a unit from the 16 and 32 cell stages one can increase the percentage of cases that produce light but not the relative amount of light produced. This suggests that an interaction may take place between the ectoblastic micromeres and the M macromeres which facilitates light production.

An inducible lysin in Limulus polyphemus *with similarities to the complement system of vertebrates: variations with disease and alterations induced by cobra venom factor* in vivo. HENRY GEWURZ, DONALD JOHNSON, VANESSA BIRDSEY, JEAN LINDORFER AND ANITA GEWURZ.

Addition of cobra (*Naja haje*) venom factor (CVF) to *Limulus* plasma *in vitro* activates a lysin system, dissociable from phospholipase A activity (PAA), with remarkable similarities to the complement system of vertebrates. We now report observations of this lysin *in vivo*.

Plasma samples from 42 consecutive animals showed a mean titer of 250 units/ml, with notable individual variation (range: < 10–1000). The three most deficient animals (<10, <10, 55, respectively) selectively had < 15,000/mm^3 circulating amoebocytes (normal = 45,000/mm^3), with decreased and distorted cells recognizable in their gills, suggesting an association between decreased lysin activity and ongoing disease.

In higher vertebrates CVF is a relatively nontoxic factor (causing only transitory hypotension and intravascular hemolysis) which induces sustained suppression of hemolytic complement. Immediately following the intracardiac infusion of 500–1000 units CVF into each of 5 crabs, (1) CVF-induced lysin activity appeared in the plasma and remained detectable for 1–3 hrs, (2) the peripheral cell count dramatically decreased to 10–40% pre-infusion control values with margination, clumping and finally marked diminution in number of gill amoebocytes, and (3) inducible plasma lysin activity fell to <10% control values and remained depressed 12-24 hrs. Thus, certain features of CVF-induced interactions of vertebrates were induced in *Limulus* along with an exaggerated cytotoxic response.

Bacterial endotoxins induced similar morphologic changes, but failed substantially to alter inducible lysin levels or to induce detectable lysin activity *in vivo*. Plasma PAA and CVF-inducible PAA reactivities remained normal in each animal cited above, again displaying lack of total identity between inducible lysin and PAA.

We conclude that the CVF-inducible lysin of *Limulus* has several features in common with CVF-induced reactivities of vertebrate complement system *in vivo*, as well as *in vitro*.

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Territoriality observed in a population of Tellina agilis (*Bivalvia: Mollusca*). WILLIAM H. GILBERT.

Northern dwarf tellin clams, (*Tellina agilis*) were collected from a tidal flat at Barnstable Harbor, Massachusetts. 36 of these deposit-feeding bivalves (average length of 7 mm) were put in a shallow sediment tray (43 × 36 × 4 cm) filled with fine sand containing benthic diatoms upon which the clams feed. The tray was placed in an unshaded, outdoor water table with flowing seawater (15° ± 3° C). The tray was removed from the water table twice daily to simulate low tides.

X-ray photographs of the sediment tray were taken at regular intervals, showing the position of each clam in the population without disturbing the sediment. Analysis of the resulting dispersion patterns indicated significantly uniform spacing, with each clam maintaining a "personal space" of about 10 cm^2.

These clams lie on their left side at a depth of about 3 cm, extending their inhalent siphons onto the sediment surface to pick up and ingest sand grains. Each may become aware of its neighbors' location through contact between siphons during feeding. During an experimental period of 25 days, the clams moved around within the sediment. However, most individuals restricted their foraging activity to a given area, seldom intruding into areas occupied by their neighbors.

The major ecological significance of territoriality is that a given area is occupied, more or less exclusively, by a proprietor. Active defense is not the only means of maintaining territories; mutual avoidance between individuals can produce the same social organization (Marler and Hamilton, *Mechanisms of Animal Behavior,* Wiley, 1966).

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Radiothyroidectomy of the toadfish, Opsanus tau. A. E. V. HASCHEMEYER AND V. LAURIE.

Although the evolution of the thyroid gland appears to have been complete with the appearance of the lower vertebrates, no conclusive function for the hormone has been found for most of these forms existing today. For example, several studies have shown that thyroidectomy by surgery or radioiodine produces little or no change in oxygen consumption or growth of fish.

The present work was carried out to ascertain whether the thyroid hormone may play a role in the striking compensation of metabolism which occurs during cold acclimation of fish. Previous studies on the toadfish have shown that liver protein synthesis cold-adapted animals at 10° is 70% greater than that of control animals adapted to 20°, when both are measured at the same temperature. The effect was found to be localized at the stage of addition of amino acid residues to the growing chains, and a sensitive assay for the rate of polypeptide chain assembly *in vivo* was developed. This procedure has now been used to determine liver protein synthetic rate in animals treated with sodium iodide-I^{131} (25 microcuries/g body weight) for one week prior to a two-week acclimation period at 10°. When assayed for liver polypeptide chain assembly, these animals failed to show the increased rate characteristic of cold-adapted fish.

The fish bodies were scanned for radioactivity using a Geiger counter fitted with a lead collimator. The results showed a high concentration of radioiodine (about 50% of the injected dose) in the pharyngeal region. No significant radioactivity occurred elsewhere. Histological examination showed pycnotic nuclei in the epithelial cells of the thyroid follicles, indicative of radiation damage. Further studies are necessary to determine whether the loss of adaptive capacity in such animals can be reversed by hormone replacement.

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The acrosome reaction in spermatozoa of Lumbricus, *as revealed by negative staining.* CATHERINE HENLEY.

After negative staining with 1% phosphotungstic acid, pH 6.8, some earthworm spermatozoa isolated from the seminal vesicles undergo spontaneous acrosome reactions (see abstract for July 14 seminar, this issue). In the initial stages of these reactions, an amorphous material is extruded at the anterior tip of the spermatozoon and the structure of the acrosome complex at the proximal region nearest the nucleus is clearly apparent, including a straight, rod-like structure indenting the acrosome vesicle. In spermatozoa in which the acrosome reaction has proceeded to completion, or near-completion, the substructure of this region is no longer visible. The entire acrosome region, except for the tip, is now filled with PTA and appears as an electron-dense area. In the unreacted acrosome, electron-dense PTA does not penetrate and no internal substructure of the acrosome complex is visible, but the details of surface structure are clearly defined. An acrosome filament appears at the tip of the completely reacted acrosome; this filament is quite rigid and straight, and its length varies from *ca.* 0.71 to 1.29 microns. There is apparently no lumen within it. At its proximal end, the filament is lodged in a squared-off depression at the end of the former acrosome complex region, for approximately 1/7 of its total length. This depression is electron-dense, so that the filament stands out clearly against the dark background of PTA filling the area.

This appears to be the first report of an acrosome reaction in an oligochaete annelid, and of negatively stained reacted acrosomes of any form, although Munn and Barnes recently described and illustrated unreacted acrosomes in negatively stained spermatozoa of barnacles.

Aided by a grant from the NIH, GM 15311.

Phosphorylation of nuclear protein following fertilization in Arbacia *and* Spisula.

JOSEPH ILAN AND JUDITH ILAN.

While studying RNA synthesis in unfertilized and fertilized sea-urchin eggs we observed that when unfertilized eggs were incubated for three hours in the presence of 20 μg/ml of actimomycin D prior to fertilization, the incorporation of P^{32} into the tRNA fraction was 10% that of the control. Analysis of the phenol extracted radioactive material on sucrose gradient revealed that the peak of radioactivity did not coincide with the optical density peak of the tRNA. Further purification of the tRNA on a MAK column showed that the tRNA was not labeled. The labeled product came off the column with the wash. It is soluble in 5% trichloroacetic acid and is precipitated by the addition of 4 volumes of ethanol. The phosphate can be hydrolyzed by treatment in 1 N NaOH for 1 hour at 90° C. The fraction was solubilized by pronase. Upon chromatography phosphoserine was identified as a major P^{32} label. A minor component was tentatively identified as phosphothreonine. The label was shown to be in a nucleoprotein which was contaminating the RNA fraction upon phenol extraction. Phosphorylation of nucleoprotein is triggered by fertilization in *Arbacia* and is independent of fertilization in *Spisula.*

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The peripheral membrane system of sea urchin eggs: an electron microscope study.

SADAYUKI INOUE, GILES H. COUSINEAU, PAUL L. KRUPA AND ATTILA BUDAY.

The fine structure of the peripheral membrane system of *Arbacia punctulata* eggs was studied with an electron microscope using the pre-shadowed surface replica method. The eggs, treated or not with trypsin powder in sea water (0.5 mg/ml) for 10 min at 22° C, were half embedded in a layer of cellulose nitrate. The surface replicas of the exposed upper hemispheres of the eggs were prepared by first shadowing with platinum and carbon-coated. The specimens were then decomposed in 18 N H_2SO_4. The peripheral membranes of the eggs, from which the vitelline portion had been digested, were isolated by gentle homogenization of the eggs and by low speed centrifugation of the brei. The supernatant was then passed through a column containing small glass beads. The membranes, adsorbed on the beads, were recovered and washed with distilled water.

Egg surfaces untreated with trypsin, and thus possessing a vitelline membrane, showed an assembly of fine filaments with a thickness of about 20 Å. On the other hand, the surface of the trypsin-treated eggs was observed to be made up of filaments with a thickness of approximately 40 Å. The peripheral membranes (170–210 Å thick), isolated after treating the eggs with trypsin also seemed to have 40 Å-thick filaments on both sides. In addition to the filamentous structures disc-like subunits of 190–240 Å in diameter and 50–90 Å thick were also seen. When these observations are compared with previous findings on peripheral membranes of eggs and sperms of the purple sea urchin and of guinea pig red blood cells, the possibility arises that plasma membranes in general are made up of layers composed of 40 Å-thick filaments on both the inner and outer surfaces.

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A model for predicting the influence of colony morphology on reproductive potential in the Phylum Ectoprocta. Karl W. Kaufmann.

The three most common types of colony morphology are those which grow linearly, like a vine, those that are restricted to two dimensions and encrust their substrate, and those that grow in three dimensions as upright bushy forms. Although there are gradations from one form to another, an idealized model of each can be described with tractable mathematical equations. If the asexual budding rate is constant, the increase in number of zoids as a function of time is a linear equation for one dimensional forms, a quadratic equation for two dimensional forms, and an exponential equation for three dimensional forms. By assuming that each zoid produces larvae at a constant rate, then equations for the number of larvae as a function of time will be integrals of the colony growth rate equations. Given characteristic budding rates for each growth form, the equations show that over a short period of time, the one dimensional forms will produce the most larvae, that over an intermediate period of time, the two dimensional forms will produce the most larvae, but that given a long time span, the three dimensional form will outproduce the other two.

Among the many factors which may influence the distribution and abundance of ectoprocts, this model isolates one factor, the effect of colony morphology on larvae production. If the other factors are of lesser importance, then the model would predict that linear growth forms would be favored on substrates which have a short half-life, while substrates progressively more stable would favor the two and three dimensional forms.

T. J. M. Schopf offered advice and laboratory space. The research was supported by grants from the Hinds fund of the University of Chicago and Sigma Xi.

Nuclear localization of the sialic acid "activating" enzyme in the unfertilized sea urchin egg. Edward L. Kean and William E. Bruner.

The sialic acid "activating" enzyme catalyses the reaction: sialic acid + CTP $\rightleftarrows$ CMP-sialic acid + PPi . Evidence has recently been presented from our laboratory demonstrating that the subcellular site for this enzyme is the nucleus. In order to investigate this assignment of cellular locale further, the distribution of this enzyme between nucleate and non-nucleate portions of the sea urchin egg was studied. Nucleate and non-nucleate segments were obtained by layering washed, unfertilized eggs from *Arbacia punctulata* in sea water over 0.85 M sucrose and centrifuging for 15 min at 24,000 × g. This relatively mild procedure should minimize artificial redistribution of the enzyme that possibly could result from homogenization. The following specific enzymatic activities (μmoles CMP-sialic acid formed per milligram protein per hour) were [mean ± S.E.M. (number of experiments)]: whole eggs, 0.174 ± 0.011 (7); nucleate half, 0.625 ± 0.13 (6); non-nucleate half, 0.167 ± 0.023 (6); pigment and yolk, 0.0356 ± 0.0093 (5); soluble, 0.59. The nucleate area was highly uniform, containing over 95% nucleate segments. The non-nucleate area was not homogeneous and contained from 3 to 10% of the initial amount of whole eggs. The yields of enzyme from these areas were (%): nucleate half, 54.4 ± 6.3 (6); non-nucleate half, 10.5 ± 4.3 (6); pigment and yolk, 1.84 ± 1.1 (5); soluble, 9.3. These observations are consistent with previous findings that the enzyme is located in the nucleus of the cell, and can be regarded as additional evidence supporting this contention. In contrast to the mammalian enzyme, the enzyme in the sea urchin egg is very labile, losing about 80% of its activity after storage at 4° C for 24 hrs.

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Aggregation and inhibition of aggregation of amebocytes of the horseshoe crab Limulus polyphemus. Dianne M. Kenney, Frank A. Belamarich and David Shepro.

When hemolymph is withdrawn from *Limulus polyphemus,* the amebocytes rapidly aggregate. Rate and extent of aggregation can be measured photometrically by recording changes in turbidity as per cent transmittance.

Amebocyte aggregation is markedly inhibited by high concentrations (60–80 mM) of buffered EDTA, and the addition of equivalent concentrations of calcium and/or magnesium do not reverse EDTA inhibition. A "serum" can be prepared by centrifugation of hemolymph in which the cells are allowed to aggregate for a minimum of 15 minutes. This serum, even when diluted 200 times, is capable of restoring full aggregation to EDTA inhibited amebocytes. A supernatant made from an homogenate of amebocytes in 0.51 *M* Tris-buffered NaCl separated under conditions which minimize aggregation acts in a similar manner. The data indicate that the active substance in serum responsible for reversing EDTA inhibition is released from amebocytes during aggregation. This active substance is non-dialyzable and heat labile, although heat treated cell homogenates retain some activity. Separation of active material from serum and cell homogenate supernatants by gel-filration on G-75 Sephadex produces a single active peak common to both cell homogenate supernatant and serum. The material in this peak absorbs maximally at 270 nm and has an apparent molecular weight of less than 17,000.

Trypsin (10,200 BAEE units/mg) is active in concentrations of 0.0125% in reversing EDTA inhibition, but bovine thrombin at concentrations up to 20 NIH U/ml is ineffective. Tosyl arginyl methyl ester (TAMe), a competitive inhibitor for trypsin and thrombin, causes inhibition of aggregation similar to that produced by EDTA. Serum in relatively high concentrations can only partially override TAMe inhibition.

Both N-ethyl maleimide (NEM) and parachloromercuribenzoate (PMB) inhibit spontaneous aggregation of amebocytes. Serum is not capable of reversing PMB inhibition even when PMB cells are resuspended in fresh plasma. The addition of cysteine to washed PMB treated amebocytes resuspended in saline results in the formation of aggregates; the amebocytes appear to cohere without release of granules or formation of pseudopods.

Supported by grants from National Heart Institute HE 10002 and HE 05411–11.

Neural control of myocardial rhythmicity in Busycon canaliculatum. K. KUWASAWA AND R. B. HILL.

The cardiac nerve was severed near its origin from the right visceral ganglion. Silver stained sections and methylene blue whole preparations revealed no nerve cell bodies along the course of the cardiac nerve nor in the ventricle. Stimulation of the cardiac nerve at frequencies increasing from 0.5/sec to 5/sec induced the appearance of ventricular rhythmicity correspondingly increasing in rate and amplitude. Tension was recorded from portions of the ventricular muscle and reached a maximum of 5 g. The resting potential of impaled ventricular fibers amounted to some 50 mv. IJP's of an all-or-none character (estimated synaptic delay 10 to 20 msec) appeared when the cardiac nerve was subjected to stimulation at threshold level for some (inhibitory) axons. The amplitude of individual IJP's amounted to 0.5 to 3 mv and at frequencies of stimulation greater than 3/sec they clearly showed summation resulting in a hyperpolarization. When nerve stimulation was stopped, the summated hyperpolarization was followed by a rebound depolarization. Stronger stimulation of the nerve produced a mixed effect, with hyperpolarization giving way to depolarization. The depolarization could lead to a local depolarizing response or to an action potential with an accompanying contraction. Single contractions could be induced by single strong shocks to the cardiac nerve, but also appeared by rebound at the end of trains of lesser shocks (hyperpolarizing during the train). Afferent activity appeared in the cardiac nerve in response to the application of 5 to 10 g tension to the ventricle, or during spontaneous beating. One might then see a temporal sequence in which the ECG first appeared, to be followed by a contraction in the course of which increased afferent activity built up and then diminished in the cardiac nerve.

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Tail resorption in Ascidians: Effects of Cytochalasin B. JAY LASH, RICHARD A. CLONEY AND RONALD R. MINOR.

During tail resorption in *Amaroucium constellatum* the caudal epidermis becomes contractile; this is associated with the alignment of 50 to 70 Å cytoplasmic filaments in each contracting cell. In *Boltenia villosa* filaments become aligned in the notochordal cells during tail resorption, but not in the epidermis. Cytochalasin B has been shown by T.

Schroeder and others to selectively disperse the filaments and to disrupt the morphogenetic processes associated with the filaments.

Nine species of ascidians were used in experiments with cytochalasin B. The following concentrations (μg/ml sea water) prevent the initiation of tail resorption in the species listed: *Distaplia occidentalis* (0.25–0.50), *Diplosoma macdonaldi* (0.25 0.50), *A. constellatum* (0.5 1.0), *Ciona intestinalis* (1.0 2.0), *Perophora viridis* (1.0–2.0), *Botryllus schlosseri* (5.0–7.0), *Styela partita* (5.0–10.0), *B. villosa* (5.0–10.0), *Molgula citrina* (10.0–15.0). The same concentrations block tail resorption once the process has begun. The effect on tail resorption is reversible if specimens are washed immediately after the tails stop moving into the trunk and if the concentrations are minimal. After prolonged treatment and washing, animals continue metamorphosing, but retain the larval tail. The smooth and cardiac muscle of the trunk and the striated muscle of the tail are not affected by the drug.

A. constellatum, B. schlosseri and *M. citrina* are viable for periods up to 7 days at concentrations of 10 μg/ml, although they are abnormal in appearance and do not increase in size. Ultrastructural analysis shows that in cytochalasin B treated animals the distribution and organization of the filaments is altered; finely granular material is found where filaments are located in controls. Specimens that have recovered from the effects of the drug have well organized arrays of filaments.

Supported in part by NSF grant GB 5394 (R. A. C) and USPHS HD 00380 (J. L.).

Reversible de-aggregation of alpha-crystallin from the ocular lens of the smooth dogfish Mustelus canis. SIDNEY LERMAN AND ELSA PFENINGER.

Alpha crystallin, one of the three soluble protein fractions of the ocular lens is a large polymer (molecular weight $0.8\text{–}1 \times 10^6$) composed of forty or fifty subunits. The de-aggregation of alpha crystallin in 8 *M* urea and the subsequent recombination of the subunits when the urea was removed was studied by means of ultracentrifugation, ORD and CD spectroscopy and by two immunochemical methods; the Ouchterlony technique and the quantitative complement fixation reaction.

Purified alpha crysallin (obtained by DEAE cellulose column chromatography) was dissolved in 0.11 *M* Tris buffer containing 0.11 *M* KCl (pH 7.7.). Ultracentrifugal analysis at 20° C, 48,000 and 60,000 rpm revealed an S_{20} value of 19.6 for the native protein. ORD studies showed a trough at 231 mμ and a peak at 205 mμ while C.D. analysis revealed a minimum at 217 mμ and a maximum at 196 mμ. Identical samples of Alpha crystallin were exposed to 8 *M* urea (by means of 24 hour dialysis) and the resulting S_{20} value was 1.0 while the ORD trough at 231 mμ disappeared. When these de-aggregated protein samples were dialyzed for 24 hours against a large volume of 0.11 *M* Tris KCl buffer (pH 7.7) the subunits apparently recombined. The S_{20} value returned to 26 and there was an apparent complete recovery of the Cotton effects both with respect to their location and magnitude.

Ouchterlony studies on the native alpha crystallin and the re-aggregated material showed complete identity while the quantitative complement fixation experiments indicated at least 80-85% recovery of the original antigenic structure following re-aggregation.

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Spontaneous miniature synaptic potentials and quantal release of acetylcholine in skate electroplaques. HENRY A. LESTER.

Miniature synaptic potentials (min. PSPs) were recorded with intracellular microelectrodes in cup-shaped skate electroplaques. Cobra toxin and d-tubocurarine blocked both PSPs (evoked by stimulating presynaptic nerves) and min. PSPs. Tetrodotoxin blocked PSPs but not min. PSPs. Min. PSP frequency increased with increases in the KCl concentration of the bathing solution. These observations suggest that the min. PSPs arise from acetylcholine packets spontaneously released from presynaptic nerve terminals. Normal PSPs had a significantly shorter time course than min. PSPs, because depolarizations greater than 10 mv activate a membrane conductance increase. When reduced by curarizing agents or by reduced calcium concentration ($[Ca^{++}]$), PSP time courses approached those of min. PSPs.

The input resistance (R) of electroplaques for small depolarizations was measured with one intracellular electrode for current injection and a second for potential recording. Min. PSP amplitude varied linearly with R, reaching 300 μv at 50 kΩ (the highest R measured). Min. PSPs did not usually appear above the electrode noise for R less than about 20 kΩ. A single min. PSP thus represents a peak inward current of about 6 nA; similar values occur at myoneural synapses.

In the range 0.4–1.0 times normal [Ca^{++}] (5 m*M*), PSP amplitude varied as the fourth power of [Ca^{++}]. This result agrees with vertebrate myoneural and squid synapses. At the lower [Ca^{++}] values, PSP amplitude fluctuated in accordance with the quantal release hypothesis. The coefficient of variation of the PSP amplitude distribution was determined for two different synaptic inputs (specified by stimulating different presynaptic nerves) to an electroplaque at 0.4 times normal [Ca^{++}]. This procedure gave quantal sizes of 26 μv and 27 μv; measured min. PSP amplitudes were 76 μv in normal Ca^{++} and 44 μv in the test solution. These discrepancies may arise from electrical cable properties of the electroplaque, from non-Poisson PSP statistics, or from giant min. PSPs consisting of several fused quantal packets. One cannot yet conclude that the observed spontaneous min. PSPs constitute the same population which superimpose to form the evoked PSP.

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A biochemical comparison of ciliary and flagellar axonemes from the bay scallop, Aequipecten irradians. R. W. LINCK.

Dynein, the axonemal ATPase of cilia and flagella, has been characterized by Gibbons. Low ionic strength dialysis or salt extraction of *Tetrahymena* cilia produce 14S and 30S dyneins with certain enzymatic differences. 14S dynein has a molecular weight of 600,000; 30S dynein can only be broken down by alkali or brief trypsinization. Reconstitution of 30S (but not 14S) dynein indicates that it comprises the "arms" of the microtubules. Only 14S dynein can be obtained from echinoid flagella. Possible biochemical and structural differences between cilia and flagella of the same species have now been investigated in *Aequipecten.* Cilia were obtained from excised gills using three different deciliating media: 70% glycerol, twice concentrated sea water, and 10% ethanol-10 m*M* $CaCl_2$. After removal of membranes with Triton X-100, all procedures yield axonemes of identical ATPase specific activities. Flagella were prepared by detailing whole sperm. Flagellar and ciliary axonemes were then dialyzed extensively under identical conditions: 1 m*M* Tris, 0.1 m*M* EDTA and 0.1 m*M* ATP, pH 7.8. In cilia one half of the original ATPase remains bound to the axonemes after dialysis and can only be solubilized by brief trypsinization. Most of the other half is solubilized during the first day and sediments as a 14S component. The B-tubules and one of the central pair also dissolve, leaving the A-tubule *singlets* held together in the circular 9-fold configuration by 1000 A periodic linkage fibers. In cross section the outer arms are gone. On SDS-acrylamide gels dynein migrates as two closely spaced bands with molecular weights of approximately 260,000 and 290,000 ± 10%. Flagellar axonemes behave differently, breaking apart on dialysis into unlinked *doublet* outer fibers from which the central pair and most of the ATPase is removed. Flagellar dynein sediments as a 14S particle and on SDS-acrylamide gels appears as two components with molecular weights comparable to ciliary dynein.

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A linear relationship between light-induced current and flash intensity in Limulus *ventral photoreceptors.* JOHN E. LISMAN AND JOEL E. BROWN.

Single photoreceptor cells in *Limulus* ventral eye were impaled with two micropipettes and voltage clamped at resting potential. We measured clamping current evoked by 12 msec. flashes of light. The cell was allowed to dark adapt between flashes. At threshold (*i.e.,* the flash intensity which evokes one "quantum bump" on the average), the average peak current is on the order of 5 na. If the intensity of the flash is increased four times, the peak clamp current increases approximately four times. With successively brighter flashes, the linear relation between flash intensity and peak clamp current holds over a 100-fold range of intensity. That is, the clamp current at 2 log units above threshold appears to be the linear summation of the current from 100 "quantum bumps." At higher light intensities, the peak current begins to saturate. However, between 2 and 3 log units above threshold, we can find a time on the rising edge of the response such that at all times preceding it, the response at one half the intensity evokes one half the current. At later times, the dimmer light evokes more than half the current.

Thus, for stimuli up to 100 times threshold, the peak light-activated current is the linear summation of quantal events and moreover, for brighter lights, this linear summation holds for early times during the response, up to at least 1000 times threshold. In addition, we postulate that some mechanism of gain reduction is responsible for the nonlinearity at later times. The latency of this gain reduction decreases with increasing light intensity.

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Large-scale electrophoretic studies of allelic variation in Mytilus edulis. ROGER MILKMAN AND LARRY D. BEATY.

In a survey of about 4000 *Mytilus edulis* in 24 samples of over 100 individuals each, mostly on or near Cape Cod, allele frequencies were determined relating to three distinct allozymes of leucine aminopeptidase by the Adamkewicz electrophoretic method.

The frequency, p, of the S allele varied considerably over space; the remainder contained M and F in a fairly constant ratio of 2:3. Six southwestern populations at varying depths (intertidal to 60 feet) and salinities, in a 100 sq mi area including Woods Hole and several islands, were quite uniform, with p ranging between 0.53 and 0.59. Just northeast of the Cape Cod Canal, p varied from 0.16 to 0.30 over relatively short distances. Intermediate locations, both on the outer Cape, as well as in the Canal and south of it, ranged from 0.34 to 0.54. The possibility of an East Coast cline for S is eliminated by the finding of 0.25 for its frequency in Salisbury, Massachusetts (near New Hampshire), and 0.15 at Sunken Meadow, on the North Shore of Long Island. A substantial deficiency of heterozygotes was found in almost all populations; this observation and direct evidence suggest the presence of a "silent" allele in addition to the other three.

Just south of the Canal juveniles (0.30) differed strikingly and highly significantly from large, mature mussels of the same sample (0.44). Fluctuations in p in intermediate locations, even closely spaced and similar ones, seem comparatively high, though few pairs of samples are significantly different. Aside from sampling error, local differences in selection *intensity* might cause a jagged distribution in any transitional zone where individuals are of diverse geographic and genetic origin, as undoubtedly here.

Modiolus demissus, the ribbed mussel, has a remarkably similar LAP polymorphism, though all three allozymes move much faster than those from *Mytilus edulis*.

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Luminescence and related fluorescence in coelenterates. JAMES G. MORIN AND GEORGE T. REYNOLDS.

The bioluminescent emission of many coelenterates involves a transfer of energy from the excited state of the emitting molecule (peak emission 460 to 485 nm) to a second, fluorescent molecule which has an emission from its excited state at 508 nm. These have been termed the photoprotein and the green fluorescent protein, respectively. The fluorescent material can easily be observed with a compound microscope using a high intensity tungsten (or mercury) light source, a blue BG 12 (Leitz) excitation filter between the light and specimen, and a Wratten No. 12 gelatin filter cut to fit into the microscope eyepiece(s). The observed *in vivo* fluorescent spectrum matches closely the *in vivo* luminescent spectrum in those species examined: *Obelia geniculata*, *O. commisuralis*, *O. bicuspidata*, *Clytia edwardsi*, *Renilla köllikeri* and *Ptilosarcus guerneyi*. The hydroids examined show either discrete green fluorescent cells (photocytes) scattered within the pedicels, stems and stolons of the colonies (*Obelia geniculata*, *O. commisuralis*, *Clytia edwardsi* and *Campanularia calceolifera*) or photocytes concentrated at the tip of the pedicel just below the hydranth (*Obelia bicuspidata*, *O. longissima* and *Lovenella gracilis*). The hydromedusae show concentrations of fluorescent cells in the tentacular bulbs (*Aequorea, Phialidium* and *Obelia*). The pennatulids show dense aggregations of photocytes within the autozooids either in the lateral portions of the tentacles (throughout in *Ptilosarcus* and proximally in *Renilla*) or in the column near the oral disc (*Stylatula elongata*).

By simultaneous application of fluorescence microscopy and image intensification these green fluorescing cells have been shown to be the exclusive site of luminescence in those animals tested: *Obelia geniculata, O. commisuralis, O. bicuspidata, O. longissima, Clytia edwardsi, Aequorea forskalea* and *Renilla köllikeri.* All green fluorescent cells were luminescent and all luminescent cells were fluorescent. There are usually one or more long fluorescent processes in the hydroids with scattered photocytes. Image intensification and concomitant fluorescence of such cells shows that all of the fluorescent areas, including the fine processes, are luminescent. These observations tend to support the generalization that the green fluorescent cells in luminescent coelenterates are the site of the luminescence.

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Evidence for glucagon biosynthesis and participation of a precursor protein in islets of the anglerfish (Lophius americanus). BRYAN D. NOE AND G. ERIC BAUER.

Glucagon biosynthesis was investigated in the islet tissue of the anglerfish, *Lophius americanus.* Glucagon was identified in TCA-precipitable, acid alcohol extracts of islets by bioassay, immunoassay, and elution rate after gel filtration. By incubating islets with ^{14}C- and ^{3}H-labeled amino acid pairs, it was shown that the proteins incorporating tryptophan (which is present in anglerfish glucagon) eluted at different rates on gel filtration from those labeled by proline, cysteine and isoleucine (which are incorporated into anglerfish proinsulin and insulin).

After 0 to 60 minutes of *in vitro* incubation of islets with tryptophan, the acid alcohol extracts contained only one peak of radioactivity on gel filtration. This peak eluted behind the front protein (BSA marker), but preceded proinsulin, indicating a peptide of 9000 or more in molecular weight. Only a small amount of the total protein from the islet extracts eluted in this (post-BSA) region. After two to six hours of incubation with tryptophan, a second major peak of radioactivity appeared. This peak eluted in approximately the same fraction as mammalian glucagon markers. Glucagon radioimmunoassay of column eluates showed that most of the anglerfish glucagon antigenicity was in the region of the second radioactive peak. Incorporation of tryptophan into these proteins was markedly inhibited by cycloheximide, a specific inhibitor of protein synthesis at the ribosome.

The post-BSA (more rapidly-labeled) protein was treated with trypsin. Rechromatography showed a decrease in radioactivity in the initial peak, with the appearance of two new peaks, one of which eluted near the mammalian glucagon marker.

Rigopoulou and others have described a large molecule with glucagon immunoreactivity in extracts of mammalian pancreata. Our data suggest that a similar molecule exists in anglerfish islets and that it may serve as a precursor protein (proglucagon).

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Morphological and electrical features of some large neurons in abdominal ganglia of Limulus polyphemus *Linnaeus.* RICHARD A. NYSTROM, RICHARD A. LEVY AND IRVING NADELHAFT.

A cluster of large somata appear at the anterior corner of each abdominal ganglion in *Limulus.* Contrary to the constant morphology and physiology found among neurons in central nervous systems of some molluscs, annelids, and other arthropods, these somata show variance in number (7–11), size (100–250 μ diameters), and relative positions within the clusters. Intracellular injections of Procion dye reveal neurons with two different branching patterns: the axons of one type exit from the ganglion through the contralateral anterior root nerve while axons of the other type course posteriorly through the ipsilateral connective. Individual neurons have different patterns of input connections from the periphery. Soma membranes possess varying electrical properties. Resting potentials range between 10 and 75 mv

(Mean: 33 ± 12 mv). Most cells impaled by an intracellular microelectrode are electrically silent; these same cells have their processes confined to the ventral nerve cord and are probably interneurons. Other cells display long (12 msec) overshooting spikes (> 30 mv) while still others display small (< mv) spikes which, apparently, have not invaded the impaled soma. Some spikes, either small or large, are followed by large hyperpolarizing undershoots. Synaptic potentials are not recorded from these large neurons but are recorded from smaller somata located elsewhere within the ganglion. Concentrations as high as 10^{-3} *M* of acetylcholine, glutamate, epinephrine, 5-hydroxytryptamine, 3-hydroxytryptamine, and gamma-aminobutyric acid have no effect on electrical parameters when applied directly to soma membranes. Correlations between electrical and morphological properties are limited by the cellular variance within this arachnid species.

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Differences in blocking frequency of presynaptic terminals of an axon innervating different crayfish muscles. I. PARNAS.

A common exciter axon (number 2) innervates the medial and lateral bundles of the deep abdominal extensor muscles of crayfish. This axon evokes large epsps in the medial bundle (DEAM) and relatively small epsps in the lateral bundle (DEAL). The DEAM generates spikes and twitches to single stimuli to this axon and contraction is fully activated by stimulating at 20/sec. The DEAL requires higher frequencies (40–50/sec). Intracellular recording simultaneously from both muscles shows that activity in DEAM is blocked rapidly on stimulating the axon at 40/sec while activity in DEAL persists at 80/sec. Recovery in DEAM to single pulses is rapid. Extracellular recordings from nerve terminals show that block in DEAM is due to failure of invasion of the spike into the fine branches. Only some branches are blocked at 20–30/sec, but all are blocked at 40/sec. The block is not due to exhaustion of transmitter since reduction of epsp's by increasing Mg to 50 m*M* does not change the blocking frequency. This finding also excludes secondary effects of the transmitter on pre- and postsynaptic membrane. It appears that the nerve terminals to DEAM have zones of lower safety factor than do those in DEAL.

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Image intensification and magnetic tape recording system for microscopic observations of bioluminescence and fluorescence. GEO. T. REYNOLDS AND PAUL BOTOS, JR.

We have previously reported numerous applications of an image-intensifier–microscope system to studies of bioluminescence. The conventional camera used to record previous observations has been replaced by a plumbicon TV camera, which provides a real time display on a monitor while recording on one inch magnetic tape. Provision is made for real time audio recording of data parameters and procedures. Instant replay of an event is possible and successive single frame or slow motion analysis can be made. With a 25 × objective in the microscope, spatial resolution of better than 1 micron is achieved. The plumbicon camera provides 16 millisecond time resolution for dynamic processes. The decision to use a plumbicon rather than a vidicon was based on several factors. The spectral response of the plumbicon provides a better match to the P11 output phosphor of the image intensifier than does that of the vidicon; there is negligible dark current; target readout exhibits a fast response; a larger target field than that of the vidicon permits a more efficient optical coupling; the gamma is close to unity, an important factor in evaluating intensity of light output; single electrons from the cathode of the image intensifier can be recorded when desired.

The method has been applied to systematic studies of bioluminescence and related fluorescence in a variety of systems and has provided information on facilitation, fatigue, duration and propagation of the light, in most instances not visible to the unaided eye and not sufficiently intense for direct photographic recording.

Supported by AEC contract AT (30-1)-3406.

Adaptation properties of squid photoreceptors. Harris Ripps and Irwin M. Siegel.

The cephalopod retina lacks many of the neuronal elements found in vertebrates, *i.e.*, horizontal, bipolar, amacrine and ganglion cells; thus the graded negative potentials recorded across a small piece of squid eyecup in response to light result primarily from excitation of the retinal receptors. These potentials were used to measure changes in retinal sensitivity during exposure to steady backgrounds of various intensities, and during the course of dark adaptation when the background illumination was extinguished.

In light adaptation, thresholds rose monotonically until a stable level was reached; with dim backgrounds this process was rapid, and the dark-adapted threshold was quickly re-established when the background light was removed. Intense light adaptation prolonged both phases; more than 15 min was required for the increment threshold to reach a stable level, and subsequent dark adaptation required about 2 hr for the complete return of sensitivity.

Since the rise in threshold produced by relatively dim backgrounds cannot be attributed to a reduced concentration of visual pigment, there is the possibility that depolarization of the receptors is maintained during light adaptation, and is solely responsible for the observed changes in sensitivity. That this is not the case was shown in an experiment in which responses were elicited by pairs of brief flashes differing in brightness by 0.6 log unit, delivered in alternating sequence, and covering an intensity range of about 5.5 log units. In all cases the inter-flash interval was sufficient to allow the D.C. level to return to its resting potential; nevertheless, the effect of the brighter flash on the dimmer was to markedly reduce the amplitude of the latter.

In the course of some of these experiments it was found that the dark adapted retina produced a biphasic electrical response to an intense flash of light; a positive deflection with a latency of about 5 msec preceded the usual vitreous negative potential. After intense light adaptation which temporarily suppresses the negative potential, the positive transient was revealed as the leading edge of a sustained positive wave the origin of which is unknown.

This study was supported in part by USPHS grants (EY-35,004, EY-00285, and EY-18766) from the National Institutes of Health.

Na currents and G_{Na} changes during spike electrogenesis of eel electroplaques. Francisco Ruiz-Manresa.

Normal spike electrogenesis in eel electroplaques occurs primarily as a consequence of a transient change in G_{Na} (activation–inactivation cycle). The change in membrane voltage so produced, induces changes in G_K (depolarizing K inactivation). Depolarizing K inactivation can be blocked by inducing complete pharmacological K inactivation applying small amounts of $BaCl_2$. The changes in membrane conductance during spike electrogenesis in this condition, are only those of the G_{Na} system, that operates in parallel with the linear G_L. The values of G_L, E_K and E_{Na} can be easily obtained from I–V plots; C_m can be calculated from the time constant of the membrane. With these values, records of membrane action potentials and dv/dt permit the calculation of the time course of I_C, I_L and I_{Na}, the sum of the three (I_m) being zero. From the calculated values of I_{Na}, the time course of the change in G_{Na} can be obtained. Spikes elicited at different temperatures show that the rise of G_{Na} is almost unaffected by the changes in temperature while its decay (Na inactivation) has a large temperature dependence, confirming previous findings obtained with different techniques.

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Temporal characteristics of pacemaker inhibition in isolated hydranths of Tubularia. NORMAN B. RUSHFORTH.

Electrical stimulation of the DOS, a conducting system which courses through the stalk and hydranth of *Tubularia*, inhibits spontaneous firing in two pacemaker systems of the polyp, the NP system in the distal stalk region and the HP system in the hydranth. Inhibition of the HP system and pacemaker systems in the proximal and distal tentacles and in mature gonophores connected to the hydranth, results from activating the DOS in isolated hydranths. In such preparations the hydranth has been surgically excised from the stalk to remove NP input to the HP system.

Inhibition of the HP system in isolated hydranths stimulated every 20, 10, 5 or 2.5 secs for 5 minute periods has the following properties. (1) Maximal inhibition occurs between 1 and 2 seconds following a stimulated pulse in the DOS. (2) The degree on inhibition increases with the frequency of stimulation. (3) During the 5 minute period following inhibitory stimulation the HP System fires at frequencies significantly greater than prestimulation levels. The level of post-inhibitory excitability is positively correlated with the degree of inhibition during the stimulation period. (4) Complete recovery from inhibition occurs between 12–16 seconds after DOS stimulation.

Inhibition of HP's in isolated hydranths is less complete but longer lasting than inhibition of the NP System in intact polyps.

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Semilunar and other factors influencing hatching from egg-masses of Melampus bidentatus *in the field and in the laboratory.* W. D. RUSSELL-HUNTER, MARTYN L. APLEY AND R. DOUGLAS HUNTER.

Populations of the snail *Melampus* inhabit higher levels of salt marshes and for adaptive reasons egg-laying, hatching and larval settlement are each confined to cycles of about four days in phase with spring tides. In egg-laying, the semilunar periodicity is an obligate process: stocks of *Melampus* brought into the laboratory maintain the summer semilunar pattern without tidal stimuli. In the field another event synchronized with spring high tides is the hatching of free-swimming veligers, which could be obligate (with a rigorous developmental timetable) or facultative (dependent on the incidence of tidal submergence).

Laboratory development at 18° C involves first cleavage (5–7 hours after laying), early blastula (12 hours), retained trochophore (4 days), and well-differentiated, active veliger (11 days). In the field, most egg-masses hatch at about 13 days, during spring high tides, yielding enormous numbers of planktonic veligers. In the laboratory, time of hatching proves potentially more flexible, and hatching of veligers can only take place when egg-masses of appropriate age are flooded with seawater. When, after 48 hours of flooding, the terminal age lay between 11.5 and 15 days (both 18° C and 25° C experiments), *universally* $> 90\%$ hatching was achieved. At 18° C, the earliest recorded masses yielding $> 90\%$ hatching were at 10.3 days, and the oldest yet recorded yielding 80% at 42 days. In another series of "tidal bathing" experiments (1 hour flooding at intervals of 12.5 hours with 18° C stocks), first and second floodings had no overt effect and the third produced $< 5\%$ or *no* hatching regardless of age. The fourth flooding was usually completely effective at all appropriate ages with appreciable hatching in 30 minutes and $> 98\%$ hatching after 1.5 hours. The total time immersed in these "tidal" experiments was only 4.5 hours (corresponding to field conditions), but it should also be noted that the elapsed time in *all* the successful experiments was about 48 hours.

Semilunar field hatching is not obligate. Hatching has flexibility (age 10–24, even 42, days), but must usually result from a *sequence of about four floodings in under 50 hours.* Being facultative, the process allows better survival and overlap of cohorts but it also re-establishes the synchronization. This is highly significant for later synchronous settlement into appropriate salt-marsh levels.

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A natural experiment in the population genetics of a marine ectoproct suggesting changes in gene frequencies with changes in temperature. Thomas J. M. Schopf and James L. Gooch.

Gene frequencies in colonies of encrusting *Schizoporella unicornis* were determined by electrophoresis of proteins on polyacrylamide gels, with subsequent staining of gels for particular enzymes. From 29 to 47 colonies were collected from 0.5–3 m depth at 5 localities spread over a linear distance of approximately 33 km. From east to west, the localities are (1) Green Pond, about 10 km east of (2) the Marine Biological Laboratory (MBL) Vineyard Sound dock, (3) Sheep Pen Harbor about 1 km from the MBL dock across Woods Hole passage, (4) Robinsons Hole about 13 kms southeast of Woods Hole, and (5) off Cuttyhunk Island, about 10 km southwest of Robinsons Hole.

Eight loci were clearly identified in four enzyme systems. One locus, leucine aminopeptidase 3, is biallelic at each locality. The frequency of the faster moving allele is higher in the west: 0.25, 0.39, 0.40, 0.69 and 0.65. The frequency of the faster allele also is higher in samples dredged from deeper, cooler water in Vineyard Sound west of Martha's Vineyard: 0.50. Summer water temperatures are highest in the east and become lower in the west. Relative to Green Pond, the temperature difference is approximately 1° C lower at the MBL dock and Sheep Pen Harbor, 4° C lower at Robinsons Hole, and 5° C lower at Cuttyhunk. *S. unicornis* grows and breeds in the summer, and is dormant through the winter.

Supported by a grant from the Block Fund, University of Chicago, to Thomas J. M. Schopf.

Effects of 1-methyl adenosine on isolated gonads and oocytes of the starfish; evidence for an ovarian wall nucleosidase. Allen W. Schuetz.

The effects a 1-methyl adenosine and 1-methyl adenine on the processes of follicle cell disintegration and oocyte shedding and maturation (germinal vesicle breakdown) were compared following *in vitro* incubation with starfish gonadal fragments or cellular components. Both 1-methyl adenine and 1-methyl adenosine stimulated shedding, maturation and follicle cell disintegration when added to intact gonadal fragments. The 1-methyl adenine was 10–100 times more effective than 1-methyl adenosine in initiating these changes. Isolated oocytes incubated in calcium free sea water however matured only in response to 1-methyl adenine. Addition of ovarian wall tissue to dishes containing 1-methyl adenosine and immature oocytes produced maturational changes in the oocytes. The incidence of maturation in the isolated oocytes was dependent upon the amount of 1-methyl adenosine present in the dish and these changes occurred first in the area of the gonadal tissue. Ovarian tissue in the absence of 1-methyl adenosine did not induce oocyte maturation. Following the incubation of ovarian wall homogenates with 1-methyl adenosine, aliquots of this mixture produced maturation in isolated oocytes. Ovarian homogenates boiled or treated with proteolytic enzyme (pronase) prior to incubation with 1-methyl adenosine were ineffective in stimulating oocyte maturation. The capacity of 1-methyl adenosine to initiate maturation in isolated oocytes appears to depend upon the ovarian wall for converting 1-methyl adenosine from an inactive to an active product. It is suggested that the ovarian wall tissue(s) contains an enzyme (nucleosidase) which converts 1-methyl adenosine to a biologically active substance.

These studies were supported in part by funds from The Population Council (M70-054c) and the Ford Foundation.

Physiological adaptations of Fundulus *embryos to varied oxygen tensions.* D. R. Shanklin and J. C. Shanklin.

Armstrong-Child stages 32–33 of *Fundulus* are distinguished functionally by the appearance of vagal inhibition of cardiac activity. Just prior to this the heart is sensitive to light and the oxygen tension of the gas layered over the dish at 20° C. In subdued light, barely sufficient to view heart action, the rate is inversely proportional to oxygen tension. Under air the rate is 27.8 ± 0.3 beats per 15 seconds. A 100 watt incandescent bulb at 25 cm causes a 33% increase within 5 minutes and a 100% increase by 2.5 hours. The effect is reversible and repeatable, requiring about 45 minutes for recovery. The increase is the same irrespective of oxygen tension, suggesting that light intensity establishes a specific rate threshold. Fry have rates about double that of eggs: oxygen layered about 44 per 15 seconds and air layered about 68. This sugests that heart rates increase with development, but since morphogenesis is advanced by 100% oxygen layering, and retarded by 7% oxygen layering, light and oxygen are probably independent stimuli to cardiac rate. *Yolk consumption* was approximated by applying volume formulas with the sac as a twice truncated sphere (pericardial sac anteriorly and axial embryo superiorly). 2 mm unfertilized eggs have a volume of 4.2 mm^3. Residual volumes at 288 hours were 1.63, 2.34, and 2.04 mm^3 for 7%, 21%, and 100% oxygen, respectively. Yolk consumption is thus minimal for air layered embryos at 1 cm depth. Embryos layered by 7% oxygen went to 839 hours without completion of hatching. These had the same yolk volume as the 288 hour eggs. The results indicate *Fundulus* embryos are well adapted to current aerohydrospheric conditions. Changes in oxygen tension have significant physiological effects. This work does not indicate the optimum conditions nor when the cardiac effects of oxygen or light begin.

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Influence of varied oxygen tension on fertilization and on developmental time in Fundulus heteroclitus. J. C. SHANKLIN AND D. R. SHANKLIN.

We have suggested oxygen as a driving force in evolution and embryogenesis. *Fundulus* eggs in 1 cm sea water layered by 7% oxygen in nitrogen, air, and 100% oxygen were maintained continuously at 20° C. A fourth group was kept at 2 cm under air. Fertilization in 0.25–0.30 cm sea water was 91%; layering at 30 minutes with oxygen at 1 cm had no secondary inhibitory effect. Fertilization rates at 1 cm under 7%, 21%, and 100% oxygen were 33%, 68%, and 27%, respectively. Subsequent survival of developing embryos at 200 hours was 61.5%, 95.5%, and 97.0%, respectively. 7% oxygen retarded all major morphogenetic sequences; up to Armstrong-Child stage 24 by 158% and after onset of circulation (stage 25) by 229%, or 208% totally. 100% oxygen accelerated early cell cleavage but retarded blastula formation and pre-circulatory morphogenesis (stages 21–24). Gastrulation began late but moved faster than in air controls. After stage 25 development to hatching competence took only 75% of normal time, and the total span was thereby shortened to 88%. Switching from air to oxygen layering after onset of circulation resulted in an acceleration of post-circulatory development by 26% calculated against total post-circulatory time and by 42% calculated against the period following the shift. The reverse shift at a similar point delayed hatching competence by 8–11%. Increased depth had a constant effect: pre- and post-circulatory intervals were both increased 60%. The results are compatible with fertilization and embryonic development as sensitive to oxygen tension. At constant temperature more oxygen means more rapid development overall. The effect is mainly mediated through a competent circulation suggesting a relation between environmental oxygen and the development of circulation.

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Studies of nucleolar RNA synthesis in starfish oocytes. ELIZABETH L. SHIPPEE, GIOVANNI GIUDICE AND ALLEN W. SCHUETZ.

The RNA synthetic activity of ovaries and of the nucleoli of oocytes in the germinal vesicle stage were examined in order to explore changes associated with oocyte maturation. Nucleoli from *Asterias forbesi* ovaries were isolated using the procedure of Vincent (*PNAS*, **38**: 139. 1952), except for the substitution of 0.01 *M* Tris buffer pH 7.4 with 0.005 *M* $MgCl_2$ and 0.01 *M* NaCl for distilled water as the suspension medium. The pellet of nucleoli from 10–15 grams of ovaries which had been incubated for four hours in 20 μC/ml tritiated uridine (sp. act. 25 C/mmole) was extracted with SDS, hot phenol and chloroform. The purified RNA was layered onto a 5–20% sucrose gradient and centrifuged for four hours at 39,000 rpm on a Beckman SW-40 rotor. P_{32} labelled 26S and 18S RNA from sea urchins were used as markers. TCA insoluble counts were found associated exclusively with RNA of molecular weight less than 18S.

Whole ovaries were incubated for four hours in tritiated uridine; half of these were shed with 1-methyl adenine. The ribosomal RNA of whole ovaries, mature oocytes and shed ovaries were extracted using cold phenol. Analysis of these gradients reveals that (a) the whole ovary synthesizes 26S and 18S RNA as well as large quantities of light RNA species; (b) mature eggs are synthesizing high levels of low molecular weight RNA, but no detectable amount of ribosomal RNA; and (c) the shed ovary synthesizes significant amounts of rRNA as well as many other types of intermediate and light RNA.

These data suggest that the nucleolus of oocytes in the germinal vesicle stage can stop the synthesis of ribosomal RNA, and still have associated with it active synthesis of several classes of low molecular weight RNA. Ribosomal RNA appears to be produced in the cells of the ovary with the exception of the fully grown oocytes.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant 5-T01-HD00026-09) and also supported in part by NIH HD03797-02.

Biochemical changes in the digestive gland of the fiddler crab, Uca pugnax, *following bilateral eyestalk extirpation.* David A. Snyder and Jonathan P. Green.

Eyestalk extirpation led to 100% molting by surviving animals, thus providing an absolute time scale to which biochemical events were correlated. The mean and median day of molting was thirteen days after destalking. Digestive glands were homogenized in 0.2 *M* Tris buffer (pH 7.8) and, after incubation in appropriate media, spectrophotometrically assayed for acid and alkaline phosphatases, α-amylase, trypsin, chymotrypsin, glucose, total lipid, and total protein.

Both alkaline and acid phosphatases reached a peak of activity six days after ecdysis. This may be correlated to the deposition of calcium phosphate calcospherites in the cuticle following ecdysis. Total lipid was undetectable from late stage D until ten days after ecdysis when feeding presumably resumed. A decrease in digestive gland lipid observed in late stage D may be due to the secretion of the lipoid, waxy layer of the new cuticle. Glucose reached a maximum concentration at ecdysis. It is suggested that lipid, through its glycerol moiety, may have been converted to carbohydrate (such as glycogen or glucose), which in turn was synthesized into chitin. Chymotrypsin was undetectable at all stages tested. Trypsin and α-amylase reached a peak of activity at ecdysis. The α-amylase activity was proportional to the glucose concentration. α-amylase converts α-1:4-glucosans (such as glycogen) to dextrins which then may be converted to glucose. The rise in α-amylase activity may be involved with the conversion of glycogen to chitin. If crustaceans can convert lipid to glucosans, an additional source of substrate for α-amylase would be present. The fluctuations in trypsin activity reflect general synthetic activity.

The biochemical changes of the digestive gland represent transfer of stored reserves to blood cells, thence to intra-epidermal connective tissue, and finally to exoskeleton-secreting cells rather than degeneration or loss of functional activity.

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Receptive field organization of ganglion cells in the retina of the smooth dogfish, Mustelus canis. William K. Stell, Henry G. Wagner and Myron L. Wolbarsht.

We have investigated the receptive field properties at threshold of ganglion cells in the moderately dark-adapted, apparently all-rod retina of young (16–24 inch) dogfish. Pieces of isolated eye cup (retina attached) at about 20–22° C were suffused with moist 100% oxygen and presented patterned monochromatic light stimuli through a dual-beam optical system including a grating monochromator. Action potentials were recorded extracellularly with glass-coated Pt-Ir microelectrodes inserted through the vitreous body, amplified and displayed conventionally.

Most units isolated in the ventral retina responded to a small centered spot of any wavelength with an ON-response, after a latency of about 0.5 second at threshold. Simultaneously presented annuli of inner radius $\geqq 1$ mm elevate the threshold for the ON-response or inhibit spontaneous dark activity, which is present infrequently. Such annuli alone, or diffuse light, most often evoke no response in these units, while a luminous bar of appropriate dimensions, intensity and location may evoke also OFF- and ON-OFF-responses. The excitatory and inhibitory regions are concentric and radially symmetric. The radius of the excitatory center is about 0.5–1.0 mm according to the change in threshold with the location of a small spot of fixed diameter or with diameter of a spot located in the center of the receptive field. The inhibitory region extends at least from a radius of 1.0 mm to 2.5–5.0 mm, but its full central and peripheral extent has not been determined. The spectral sensitivity of both the excitatory and inhibitory processes is identical; it is maximal at 500–510 nm but the function appears narrower than expected from corresponding pigment spectra, perhaps because of preferential reflection of light of certain wavelengths by the choroidal tapetum lucidum.

Ganglion cells with different receptive field properties have been observed occasionally but their organization is not yet understood.

Isolation of nexin—the linkage protein responsible for maintenance of the nine-fold configuration of flagellar axonemes. R. E. STEPHENS.

After detergent removal of membranes, dialysis of cilia or flagella against low ionic strength media generally results in removal of the ATPase dynein and the solubilization of the central pair and matrix, leaving outer fiber doublet microtubules connected either as a ring of nine in cilia or as a sheet of nine in flagella. The nature of the linkage between such outer fibers has proven quite elusive. Some reports describe circumferential material connecting adjacent A-subfibers while others indicate connections running from the A-subfiber of one doublet to the B-subfiber of the next. Axonemes from *Strongylocentrotus droebachiensis, Asterias* sp., and *Arbacia punctulata* sperm flagella were incubated at 40° C for 2 minutes in 1 mM Tris·HCl, pH 8.0, and 0.1 mM $MgCl_2$. Such treatment results in depolymerization of the B-subfiber, and removal of dynein, central pair, and matrix proteins. Remaining are nine singlet microtubules connected in parallel by periodic bridges of an apparently extensible material roughly 200 Å wide and spaced at 1000 Å ± 10% intervals along the tubules. In these species at least, linkage material thus occurs between adjacent A-subfibers. Depolymerization of A-subfibers by treatment at pH 3 leaves insoluble linkage material. SDS polyacrylamide gel electrophoresis of whole axonemes shows dynein and tubulin bands, a moderately strong third component, and numerous secondary bands; electrophoresis of thermally-fractionated A-microtubule-linkage material complex indicates that this third component is greatly enhanced. Electrophoresis of the material remaining after tubule removal shows primarily this third component. The protein represents about 2% of the total axonemal protein and hence less than 1% of the total flagellar protein. The molecular weight by SDS gel electrophoresis is 165,000 ± 10%. The term "nexin" is proposed for this protein, derived from the Latin *nexus,* a tie binding together members of a group.

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Species of Renicola *(Trematoda) in the kidneys of the gull,* Larus argentatus. HORACE W. STUNKARD.

Stunkard and Shaw (1931) described *Cercaria parvicaudata* n. sp., from *Littorina littorea* at Woods Hole, Massachusetts. In a later report, Stunkard (1950) gave a more complete description of the species and noted the striking resemblance between *C. parvicaudata* and a species he had described (1932) as *Cercaria roscovita* n. sp., from *Littorina saxatilis nigrolineata* taken at Roscoff on the Brittany coast of France. Other similar cercariae were discovered in *Thais lapillus,* taken near Boothbay Harbor, Maine, and on the north shore of Cape Cod. These cercariae encysted in bivalve mollusks, especially *Mytilus edulis, Pecten irradians* and *Gemma gemma* and developed to sexual maturity in the renal tubules of *Larus argentatus.* They were described as a new species, *Renicola thaidus* Stunkard, 1964. During the summer of 1965, cysts containing metacercariae of *C. parvicaudata* were fed to gulls, *L. argentatus,* but no worms were found in the kidneys and attention was directed to other parasites, especially members of the Notocotylidae.

Werding (*Marine Biology*, **3**: 306–333, 1969) reported that *Cercaria roscovita* from *L. littorea*, taken on the coast of the North Sea near Helgoland, developed in the kidneys of *L. argentatus* to mature worms that he described as *Renicola roscovita* (Stunkard, 1932). Werding raised the question of identity between species of *Renicola*. It is apparent that *C. parvicaudata* is a species of *Renicola* and the reasons for the earlier failure to complete the life-cycle are not clear. The experiments are repeated, but since some two months are required for the worms to mature in the renal tubules, final results are not available. Since *C. parvicaudata* and *C. roscovita* infect the same intermediate and definitive hosts on opposite sides of the Atlantic, their identity is probable, but the species is distinct from *R. thaidus*.

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Parallel changes in the thresholds of current induced tensions and Ca-spikes in isolated crayfish muscle fibers. G. SUAREZ-KUREZ, P. W. BRANDT AND J. P. REUBEN.

Recent evidence indicates that electrical stimuli provide Ca to the contractile system from superficial membrane sites. A parallel relation has been observed between experimentally produced changes in threshold (mv depolarization) for a Ca-activation process (procaine-induced spikes) and for just detectable tensions evoked by intracellularly applied depolarizing currents, suggesting that membrane Ca-activation is associated with excitation-contraction coupling. Tension thresholds increase linearly with increasing concentrations of Mn (1–5 m*M*). The slope is about 6 mv/m*M* Mn. The threshold for the procaine induced spikes increases by approximately 4 mv/m*M* Mn. When Ca_o is increased in the range 13.5 to 150 m*M* the thresholds for both spikes and tension increase initially with a slope of about 4 mv/10 m*M* Ca, but the slope decreases at higher concentrations of Ca. SCN (2–200 m*M*), on the other hand, decreases the threshold values for the spikes and the tension. In both cases the function is logarithmic, the thresholds decreasing as SCN is increased (−4 mv/log SCN). Thus, the changes induced by the three agents obey different functions but in each case the effects on the thresholds for activation of Ca-spikes and tension development are parallel.

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Distribution and metabolism of 1-methyl adenine in the starfish during shedding and oocyte maturation in vitro. BRYAN TOOLE, SHIRLEY HULL, ELIZABETH L. SHIPPEE AND ALLEN W. SCHUETZ.

The incorporation of ^{3}H-1-methyl adenine into ovarian fragments and into isolated oocytes of *Asterias forbesi* has been examined as a means of providing information about the action of 1-methyl adenine in inducing shedding and maturation of starfish oocytes.

Continuous uptake of isotope occurred for a period of 2–4 hours into pieces of ovary exposed to ^{3}H-1-methyl adenine in filtered sea water. As much as 60% of this isotope was incorporated after the processes of shedding and maturation were complete, and at high doses of 1-methyl adenine the amount incorporated was 20–30 times that needed for these processes to occur. The proportion of uptake into eggs and ovary wall was approx. 1:40 even though the proportion of the two components by weight is 5:1. Autoradiography performed after uptake of isotope for periods of 5–70 minutes revealed a progressive accumulation of grains over the outer epithelial layer of the ovarian wall, with virtually no grains elsewhere.

At least 90% of the isotope taken up by the ovary wall was dialysable and no isotope was recovered in hot or cold trichloroacetic acid precipitates. The dialysable material was found to have the same capacity to induce maturation of isolated oocytes as 1-methyl adenine.

The major proportion of isotope incorporated into oocytes, either isolated or within the ovary, was also found to be dialysable and no incorporation into nucleic acid or protein was obtained.

The presence of dinitrophenol prevented the shedding and maturation of oocytes by 1-methyl adenine but the uptake of ^{3}H-1-methyl adenine into the ovary wall or into the oocytes was not affected. Preincubation with dinitrophenol followed by washing did not inhibit the action of the hormone. It is concluded that either (i) the uptake of ^{3}H-1 methyl adenine in no way reflects the physiological action of the substance; or (ii) dinitrophenol is acting at a step in these processes subsequent to the incorporation of 1-methyl adenine.

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Permeability and structure of cellular junctions in the starfish embryo. Joseph T. Tupper, Karl S. Wittman and John W. Saunders, Jr.

Electrical coupling between cells of the starfish embryo, *Asterias forbesi,* arises at the 32-cell stage. This is in contrast to several other embryos which exhibit cell to cell communication much sooner, *e.g.,* between the first two daughter cells and all cells thereafter in amphibians. Coincident with the onset of cellular coupling at the 32-cell stage is the formation of surface membrane junctions between adjacent cells of the embryo. At the 16-cell stage there are no visible junctions at the electronmicroscope level and the cells are separated by rather wide gaps (1000 Å to 1 μ). Electronmicroscope observations on the cellular junctions arising at the 32-cell stage show them to possess characteristics of both tight and gap junctions, *e.g.,* 130–160 Å width, pentilaminar structure when stained after dehydration. However, due to problems of fixation of the embryos, enough detail in structure is not present to distinguish between these two types of junction, one of which is strongly implicated in electrical transmission between cells, *i.e.,* the gap junction. The permeability of these junctions to larger molecules has been studied by iontophoretic injection of the fluorescent dye Procion Yellow (MW ~500). Cells injected at the 16-cell stage have been observed to continue division to the 32-cell stage, which serves as an indication of cell integrity. However, the dye is confined to the two daughter cells, even though the embryos show electrical communication at this stage. The dye is also confined to daughter cells at the 64-cell stage. No observations have been possible beyond this stage due to difficulty in exact dye localization. It should be noted that Procion has been shown to bind membranes of the crayfish axon. Such binding could reduce its mobility across junctions. However, we have not observed any preferential localization of the dye at membranes of the starfish cells. It is interesting to speculate that such junctions may, at a later time, exhibit permeability to larger molecules and that this selectivity may play a role in development.

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DNA synthesis during regeneration in Tubularia. Kenyon S. Tweedell.

The relation of cellular activity to primordium formation during hydranth regeneration in the hydroid *Tubularia* is obscure. In order to determine the site and time of DNA synthesis during regeneration, a series of regenerates were incubated in H^3 thymidine for various intervals after hydranth amputation. In another series of experiments regenerates of *Tubularia spectabilis* and *Tubularia crocea* were sampled after isotope injection at succeeding stages of regeneration. A 1 to 2 μl quantity of H^3 thymidine (0.3 to 1 μc/stem) was injected at different stages (from 3 hours post amputation until after new hydranth formation). After $\frac{1}{2}$ to 1 hour pulses, the regenerates were fixed and examined by autoradiography.

Uptake was slight in all stages of regenerating stems when they were exposed to 1–2.5 μc/ml of H^3 thymidine in the culture fluid. Apparently the isotope cannot penetrate the perisarc unless an opening is maintained.

In the injected series of 1 hour pulse during the first 3 hours after amputation showed uniformly scattered nuclear uptake in both the epidermis and the gastrodermis. Further injections at 2 hour intervals from 4.5 to 15 hours after amputation gave relatively little labeling to any parts of the regenerates. Mass cell migration has taken place by this time.

From 17 hours after amputation, when the two banded hydranth primordium first appears in *T. spectabilis,* nuclear uptake was again evident in both the hydrocaulus and in the primordium. Thymidine uptake was found in both epidermis and gastrodermis in all regenerative stages but qualitative and quantitative differences were noted along the stem. At the proximal end, the label was more heavily concentrated in the epidermis and uptake diminished toward the distal end. The gastrodermis was only moderately labeled at the proximal end and along the hydrocaulus but uptake increased distally.

In the primordium nuclear uptake was most evident in the gastrodermis and relatively little was seen in the epidermis except during the late tentacle formation. As regeneration neared completion, the lower hydrocaulus was still labeled; in the hydranth uptake was concentrated in the basal hypostome and peduncle. Within the first day post emergence incorporation was limited to the hydrocaulus.

Absorbance melting profile analysis of DNA and chromatin in somatic cells and sperm of the spider crab, Libinia emarginata. JACK C. VAUGHN AND GERTRUDE W. HINSCH.

Recent studies have shown that crustacean nuclear DNA from a variety of tissues of various species bands in the analytical ultracentrifuge in at least three different zones, corresponding to the main band, an (A+T)-rich satellite and a (G+C)-rich satellite. The present study examines total testis DNA, isolated sperm DNA and sperm chromatin, with special reference to DNA heterogeneity and the presence of sperm nuclear basic proteins.

Absorbance melting profiles of isolated total testis DNA in SSC/10 show two distinct T_m values: 48.5° C (7.7% of the total hyperchromicity, presumably representing the (A+T)-rich satellite reported in closely-related species) and 70.0° C (88.9% of the total hyperchromicity, representing the main band DNA). An additional transition occurs at about 90° C (3.4% of the total hyperchromicity) and presumably represents the (G+C)-rich satellite. This latter transition often appears biphasic, suggesting heterogeneity, although this is not yet certain.

A technique is described for isolation and purification of sperm (99%+ pure), and for fractionation of these cells into highly purified acrosomes and chromatin. Absorbance melting profiles for sperm chromatin and for sperm chromatin treated extensively with pronase are identical, suggesting that basic proteins are not associated with sperm chromatin. Of special interest is the finding that these profiles reveal a lack of the (A+T)-rich satellite in deproteinized sperm chromatin. In order to determine if this apparent loss is due merely to the chromatin isolation technique, sperm DNA was isolated by a modification of the Marmur procedure, with identical results. It is suggested that the loss of the (A+T)-rich satellite occurs during spermiogenesis, as Feulgen photometric analysis of spermatid nuclear DNA content in another crab, *Emerita analoga,* has revealed a 6–9% loss in nuclear DNA during spermiogenesis.

We hypothesize that the (A+T)-rich satellite in crustaceans represents a highly amplified gene (or genes) which appears during development and differentiation of somatic cells. The significance of these results remains to be established.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant 5-T01-HD00026-09).

Fluorescence analysis of the excitation process in nerve using hydrophobic probes. AKIRA WATANABE, ICHIJI TASAKI AND MARK HALLETT.

A hydrophobic probe, 2-p-toluidinylnaphthalene-6-sulfonate (TNS), was injected into a squid giant axon and fluorescence properties of the axon were examined under illumination with near visible ultraviolet light (365 nm wave length). The axon was found to fluoresce with an emission spectrum spreading from about 410 to 560 nm, the emission maximum being around 450 nm. When such a TNS-stained axon was stimulated, the intensity of the fluorescent light between 420 and 480 nm was found to decrease slightly. This finding suggests that the process of nerve excitation involves a transition of the membrane from a hydrophobic to a hydrophilic state. When the incident (*i.e.*, exciting) UV light was polarized in the direction either parallel or perpendicular to the longitudinal axis of the axon, the fluorescent light contribution to the transient decrease during nerve excitation was nearly completely polarized, indicating that the axonal membrane has a rigid, crystalline structure. When the stimulation was preceded by weak anodal polarization the magnitude of the optical response was greatly enhanced. Probably anodal polarization increased the number of the probe molecules at hydrophobic sites in the axon membrane. The fluorescence intensity of TNS-stained axons was examined under voltage-clamp. With depolarizing clamping pulses, the magnitude of the transient decrease in fluorescence was found to vary with the loss of membrane impedance. Tetrodotoxin delayed the time-course and often reduced the magnitude of fluorescence changes. The significance of these experimental findings are discussed.

Synaptic organization of the oculomotor nucleus: a comparative electron microscopic study. STEPHEN G. WAXMAN AND GEORGE D. PAPPAS.

Electron microscope studies of the oculomotor nuclei of five vertebrates (*Chilomycterus schoepfi, Rana pipiens, Anolis carolinensis, Felis domestica,* and *Macaca mullata*) reveal that electrotonic synapses may be a general feature of the inframammalian oculomotor nucleus. Oculomotor neurons in the spiny boxfish (*Chilomycterus*) have one or a few large dendrites which ramify in the tegmental neuropil. A single presynaptic fiber may establish many *en passant* synapses with a postsynaptic cell, and may penetrate into indentations in the cell surface. *Rana* oculomotor nucleus contains similar motoneurons and smaller multipolar interneurons. There is a specific relationship of some axonal processes with subsynaptic cisterns. Axodendritic synapses predominate on the multipolar neurons in chameleon (*Anolis*) oculomotor nucleus. Subjunctional bodies are present. The presence of close membrane appositions in the teleost oculomotor nucleus has previously been correlated with electrotonic coupling. Close appositions are present in *Rana* and *Anolis,* so that electrotonic coupling probably occurs in these species. Presynaptic terminals at the electrotonic synapses, which often arise at nodes of Ranvier, contain both clear and dense core vesicles. In contrast, gap junctions were not found at synapses on the multipolar neurons in lateral divisions of cat and monkey oculomotor nuclei. Presynaptic terminals in these nuclei are large, and establish axosomatic, axodendritic, and spine synapses. The presence of axo-axonic synapses suggests the operation of presynaptic inhibition. Vesicle distributions at synapses in each of the species examined can be distinctly non-random. Vesicle populations often exhibit a sharp boundary located along an extension of the axon cylinder, suggesting that intra-axonal filamentous networks and/or axoplasmic flow may act as a barrier to vesicle diffusion.

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Only decreases in illumination elicit spike responses in the siphonal photoreceptor system of the hard-shell clam, Mercenaria mercenaria. MICHAEL L. WIEDERHELD AND EDWARD F. MACNICHOL, JR.

This clam responds behaviorally to dimming of light by retracting the siphon and closing the shell. Presumably this is a protective reflex used to withdraw the siphon when a predator's shadow passes over. We have recorded spike responses from axons in the siphonal nerve which could mediate this reflex.

Experiments were performed on the isolated siphon, posterior adductor muscle and visceral ganglia maintained in sea water. The siphonal nerve was cut near the ganglion and small strands were sucked into a 50 μ suction electrode. Spikes from single axons were isolated with a window circuit. Responses were obtained by illuminating the yellow-pigmented region of the inner siphon wall. Light with a spectral peak near 510 nm was used throughout, since earlier experiments showed this system to be maximally sensitive at about this wavelength.

We have observed only off-responses, never responses to the onset of illumination. Using a small shadow, the receptive area of a single axon can be localized to a region of approximately 85 μ, indicating that the receptor cells which these axons innervate probably lie near the surface of the inner siphon wall.

The off-response appears to grow monotonically with the duration of illumination, at least up to durations of 500 sec. The off-response to a flash of light is dependent on the amount of previous illumination. In a dark-adapted preparation, the first spike after a flash may not occur for 5–10 sec whereas, with an identical flash, the light-adapted latency can be less than 100 msec. The response to a standard flash can be reduced or eliminated by a steady background light. The flash response is enhanced immediately after the background is terminated and then decays back to the pre-background level. This decay time varies from a few seconds to several minutes, depending on the intensity and duration of the background.

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Structure and function of microsporidian spores. KARL WEIDNER AND WILLIAM TRAGER.

Microsporidian sporozoa are small eucaryotes suited for intracellular environments such as muscle, gut and nerve cells. They induce chronic to acute infections in most major animal categories, including man, but are particularly well known as parasites of arthropods and fish. In the genus *Nosema* the resistant spores characteristically extrude a fine filament when ingested by an appropriate host. It is thought that this filament serves to inject infective material into a host cell, but little is known as to the mechanism of extrusion or the nature of the early infective stages.

This ultrastructural study featured the spores of two species, *Nosema lophii* from the cranial ganglia of the anglerfish (*Lophius americanus*) and *Nosema* sp. from the muscle tissue of the blue crab (*Callinectes sapidus*). *N. lophii* has a spore about 3 μ long × 1 μ wide containing a conspicuous nucleus about 0.7 μ in diameter, cytoplasm with numerous free ribosomes and an elaborate polar arrangement of pleated membranes (polaroplast) that are continuous with the outer of the two envelopes of the tightly coiled polar filament. The spore wall consists of a thick (0.1 μ) electron-lucent inner layer surrounded by a denser, thinner layer (0.02 μ).

Spores of *Nosema* sp. were induced to extrude their filaments by pretreatment for 1 hr with either 0.1 *N* KOH or Michaelis veronal acetate buffer (pH 9) followed by immersion in a tissue culture medium (199 or Eagle's MEM). Extrusion reached a peak within 15 minutes. Spores with an attached extruded filament were empty except for a few membrane profiles. The extruded filaments had two envelopes with the same diameter as in the pre-extruded state.

Thus the structure of microsporidian spores fulfills two functions: (1) protection by means of the thick wall and outer coat; (2) extrusion by an explosive mechanism projecting the spore's vital contents through the polar filament into a host cell.

Supported by a grant (5T01-A1-00192) from the National Institute of Allergy and Infectious Diseases.

Effect of puromycin on the growth pattern of Campanularia flexuosa. KARL S. WITTMAN.

Growth of *Campanularia flexuosa* occurs through elongation of the stolon and development of hydranths from hydrocauli. Newly differentiated hydranths have a life span of about one week and are resorbed with the products of regression available for further colonial growth. The objective of this study was to determine the effects of puromycin on stolon elongation and the hydranth differentiation-regression cycle.

Colonies were mainained in culture on glass slides suspended in filtered sea water at 17° C and fed daily with newly hatched *Artemia* nauplii. Healthy cultures were placed in petri dishes containing either sea water alone or 20 μg/ml puromycin in sea water for periods from 10 hours to seven days.

The rate of stolon elongation of puromycin-treated cultures was significantly lower than that of control cultures. Growth rate reduction was noticeable between 12 and 24 hours after puromycin treatment was begun. Within 24 hours afer removal of puromycin normal stolon growth was resumed. The length of treatment did not influence the recovery period.

All mature hydranths of puromycin-treated cultures entered the regression phase simultaneously within 24 hours after drug administration, while controls maintained their usually low rate of hydranth regression. Treated hydranths in stages of differentiation regressed instead of completing development. No new hydranths were formed during puromycin treatment. Within 24 hours after removal of puromycin hydranth differentiation resumed at rates similar to controls.

This work suggests that puromycin acts by (1) inhibiting the process of differentiation possibly by depressing synthesis of protein and (2) reducing the immediate reuse of the products of regression.

This study was supported by a Faculty Fellowship and Grant-in-Aid from the State University of New York Research Foundation.

The choroid retes of scup, Stenotomus versicolor, *and striped bass,* Roccus saxatilis. ROBERT C. WOLLEY AND D. EUGENE COPELAND.

Two retes commonly found in teleost fishes—the swimbladder rete and the choroid rete of the eye—are involved in oxygen concentration by countercurrent multiplication. The swimbladder rete of the striped bass (*Roccus saxatilis*), scup (*Stenotomus versicolor*), and eel (*Anguilla rostrata*), as well as the choroid rete of the striped bass and scup (the eel has no choroid rete) were studied by light and electron microscopy. All three swimbladder retes exhibited cross section features characteristic of a rete mirabile: densely packed array of uniformly sized capillaries with regular interdigitation of afferent and efferent elements. The choroid rete of the bass eye was composed throughout its length of irregularly shaped, thin walled capillaries which were loosely packed and irregularly arranged. Scup choroid rete contained small, thick walled, irregularly shaped capillaries in its proximal regions. In the distal portions the capillaries assumed two different sizes, part remaining small and thick walled, the rest becoming large, irregular and thin walled. The retes of both the eye and the swimbladder are structured to provide countercurrent flow of blood. The rete of the eye, however, possesses vessels that on the average are larger, more thin walled, and more irregularly arranged than in the rete of the swimbladder. So much so that in some instances (striped bass particularly) the venous return seems to be in the form of open sinusoidal spaces. This could permit a more slow, turbulent flow of blood. The countercurrent principle would still apply but the efficiency of the system would be less. On the other hand, there would be more time for active secretory exchange(s) to occur.

This research was supported by grant in aid from NIH (GM-06836), NSF (GB-676) and training grant from NIH (GM-669).

How protein coloration modifies interactions between lenses and UV light. S. ZIGMAN, T. YULE AND G. GRIESS.

The UV light (365 $m\mu$) absorbed and the fluorescence (360 $m\mu$/450 $m\mu$) emitted by normal and artificially colored dogfish lenses and extracted proteins were compared. The untreated dogfish lens (1 cm diameter) absorbed 50% of this light, and the relative (to water) fluorescence was 64. When the absorptions and fluorescences of the purified α, β, and γ-crystallins were added up and extrapolated to their concentrations within the lens (30% protein), the values obtained were far in excess of these in the intact lens. Protein-protein interactions within the highly structured lens appear to minimize absorption and fluorescence by it. β-crystallin exhibited the greatest absorption and fluorescence of the three crystallins.

When whole dogfish lenses, homogenates or purified crystallins were exposed to 400 $\mu W/cm^2$ of 365 $m\mu$ light for 18 hrs in the presence of 0.1% tyrosine, L-dopa, tryptophane, or p-aminobenzoic acid (PABA) at neutral pH, the reagents themselves, the lenses and the crystallins were stained various shades of yellow (tryptophane), brown (PABA), and black (tyrosine, L-dopa). Lenses and crystallins were also stained during soaking in colored solutions of these irradiation products. Reducing agents (ascorbic acid, dithicorythritol) prevented such color formation in UV light, but could not reverse it. The color could not be removed from the lens proteins, which even leached it out of concentrated solutions. The same color was caused by exposure of these reagents to sunlight and fluorescent light. A

100% + increase in the 365 mμ absorption and 360/450 mμ fluorescence of whole lenses or purified crystallins resulted from reaction with tryptophane and PABA under UV light. The greatest increase was found in the γ-crystallin fraction.

The development of colored lenses in animals normally exposed to near UV light may protect the retina from the damage of irradiation by this increase in absorption and fluorescence. In the dogfish, rat and rabbit, the lens is normally colorless; in humans and certain diurnal animals, it becomes yellow to brown with aging. The formation of this color is probably a chemical adaptation in the lenses of animals exposed to near UV light (*i.e.,* sunlight) which converts some of this light into fluorescence. A lower intensity of UV light thus reaches the retina thereby reducing possible damage to cells (rods, pigment epithelium) sensitive to it.

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PHYSIOLOGY OF THE REPRODUCTIVE CYCLE IN THE COCKROACH *BYRSOTRIA FUMIGATA* (GUÉRIN)

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Previous studies on reproduction in cockroaches and in certain other insects have implicated juvenile hormone in the control of various processes occurring during the female reproductive cycle: yolk formation (reviews: Wigglesworth, 1964; Engelmann, 1968), vitellogenic blood protein secretion (Coles, 1964; Minks, 1967; Bell, 1969a; Engelmann, 1969; Scheurer, 1969a), colleterial gland activity (Willis and Brunet, 1966; Bodenstein and Shaaya, 1968; Shaaya and Bodenstein, 1969) and sex pheromone secretion (Barth, 1962, 1968; Emmerich and Barth, 1968). These processes have not been investigated, however, with regard to their integration in the reproductive cycle. It is the purpose of this communication to describe the reproductive cycle of *Byrsotria fumigata,* an ovoviviparous cockroach, and to report observations on the factors which control the initiation and termination of these four processes during the cycle.

The cycle of *B. fumigata* differs from that of the more primitive oviparous forms in that the oothecae are oviposited into a brood sac and incubated within the female until hatching rather than being produced at frequent intervals and oviposited externally (Fig. 1). Oocyte development and the other reproductive processes characteristic of the preoviposition period are inhibited during the lengthy term of pregnancy; after parturition the various reproductive processes recommence. Owing to the greater complexity of their cycle, the ovoviviparous cockroaches are more interesting than oviparous species from the viewpoint of the physiological control mechanisms operating in the regulation of the cycle. Female reproductive cycles in cockroaches have been discussed in relation to sexual behavior (Barth, 1968) and also with regard to the evolution of the cycle (Roth, 1970). The oviparous type of cycle, as exemplified by *Periplaneta americana,* has been treated by Bell (1969b) with special reference to oocyte development.

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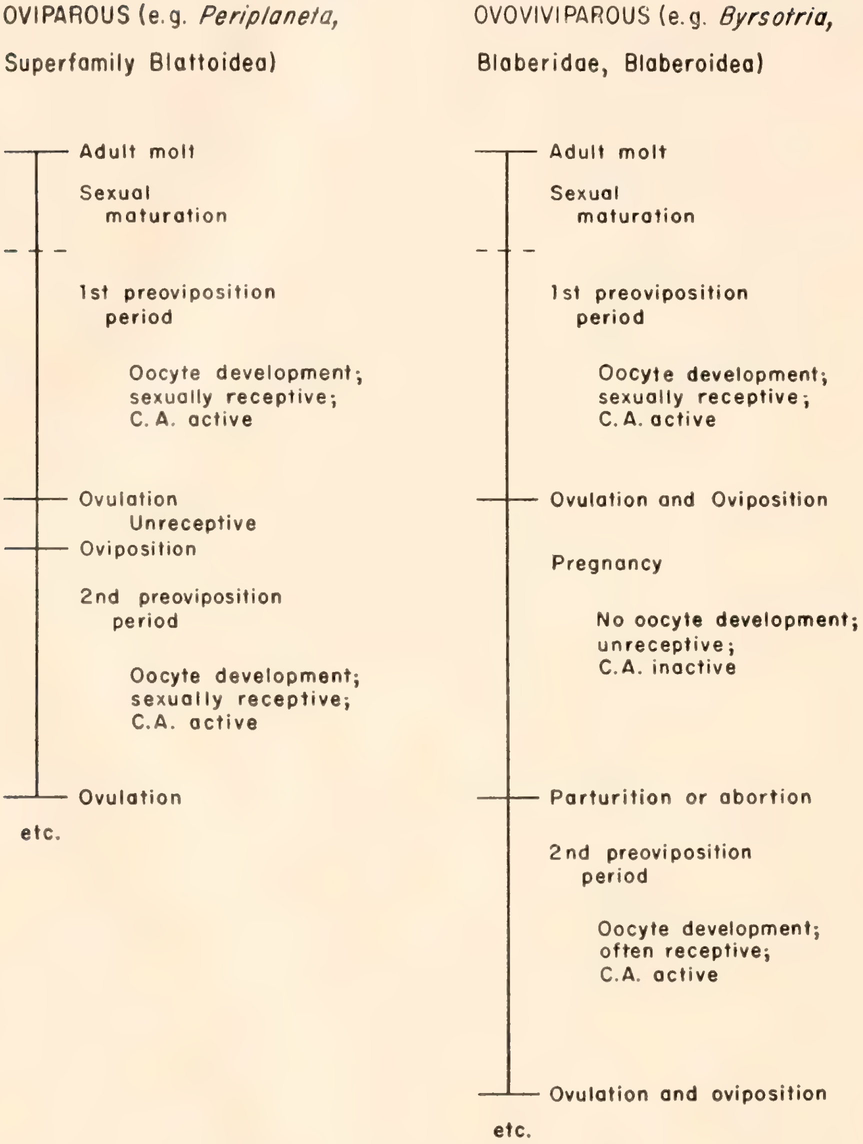
OVIPAROUS (e.g. *Periplaneta*, Superfamily Blattoidea)
Adult molt
Sexual maturation
1st preoviposition period
Oocyte development; sexually receptive; C. A. active
Ovulation
Unreceptive
Oviposition
2nd preoviposition period
Oocyte development; sexually receptive; C.A. active
Ovulation
etc.
OVOVIVIPAROUS (e.g. *Byrsotria*, Blaberidae, Blaberoidea)
Adult molt
Sexual maturation
1st preoviposition period
Oocyte development; sexually receptive; C. A. active
Ovulation and Oviposition
Pregnancy
No oocyte development; unreceptive; C.A. inactive
Parturition or abortion
2nd preoviposition period
Oocyte development; often receptive; C.A. active
Ovulation and oviposition
etc.

FIGURE 1. Reproductive cycles of cockroaches.

METHODS AND MATERIALS

1. Insect material

B. fumigata nymphs were maintained in plastic cages with access to food and water as described previously by Barth (1964). All females used in the experiments were removed from the cages within 24 hours after they emerged as adults (designated day 0) and were thereafter housed individually in 250 ml beakers. The females were mated between days 12 and 16 after emergence and spermatophore insertion was used as a criterion for successful mating. Experimental females were bled and dissected within 6 hours following the assay for pheromone, and the blood was stored at −5° C. The clotted cells were separated from the serum by allowing the serum to seep out of the clot in a horizontal test tube. Oocytes and colleterial glands were excised from dissected females and extracted as described below.

2. *Pheromone assay*

The sex pheromone assay, previously described by Barth (1961, 1962), consisted of removing a filter paper from a beaker containing a female and placing it in or suspending it a few inches above a container of males for two minutes. If the female was secreting pheromone (absorbed by the filter paper) the paper elicited courting responses in groups of adult males. In the present study an additional assay for pheromone secretion was used; the virgin female herself was placed in or held a few inches above a container of males. In this way the presence of the pheromone on the cuticle of the female could be assayed by the male response.

3. *Yolk deposition*

The criteria used to measure the initiation and progress of yolk deposition were the appearance of refractile yolk spheres in the oocyte cortex, appearance of significant amounts of extractable protein in the oocytes and changes in oocyte volume (Bell, 1969b).

4. *Vitellogenin secretion*

The Oudin (1948, 1952) technique was used to resolve the resulting antigen-antibody reactions when blood or yolk fluid was reacted with an antiserum containing antibodies homologous to the yolk antigens. The antiserum was derived from the blood of rabbits which had been injected with the proteinaceous portion of *B. fumigata* yolk fluid. The yolk extracts and the antiserum were prepared

TABLE I

Concentration of yolk antigens in cockroach blood relative to the concentration in a yolk fluid standard

	Antigen (% concentration)			
	A	B	C	D
Yolk fluid	100.00	100.00	100.00	100.00
Ovariectomized female (day 16)	4.00	10.90	86.40	100.00
Adult female (day 15)	1.20	4.30	101.05	118.00
Pregnant female	0.04	0.03	93.70	87.00
Adult male (day 15)	0.00	0.00	101.05	117.00
Female nymph	0.00	0.00	94.00	94.00

using techniques previously described for the preparation of an antiserum against yolk proteins of *Periplaneta americana* (Bell, 1970).

Glass tubes (6 × 0.5 cm) were half-filled with diluted antiserum mixed with agar (1:10). When the antiserum-agar mixture was solidified, yolk fluid or cockroach blood was layered on the tubes and a topping of mineral oil was added to prevent evaporation. As the antigens diffused into the antiserum-agar, four precipitin bands with sharp leadings edges were observed. Of the four bands, 2 were of light density (antigens C and D) and resulted from precipitin tests with yolk fluid or blood from male, female or immature cockroaches. Table I shows that the

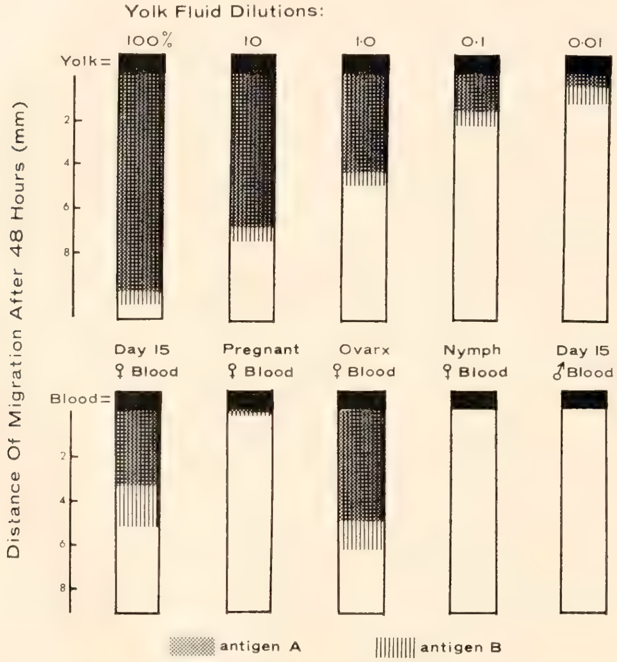

FIGURE 2. Distance of migration by precipitin bands in Oudin tubes containing antiserum absorbed with male blood and mixed with agar.

concentration of antigens C and D is similar in yolk fluid and blood from males, females and nymphs. Added to the fact that antigens C and D do not accumulate in ovariectomized female blood and are not selectively sequestered by the oocytes (Table I), these data suggest that antigens C and D are not female-specific vitellogenic blood proteins, but occur as minor components of the yolk.

Antigens A and B which produce the two dense precipitin bands, on the other hand, are taken up by the oocytes over a considerable concentration barrier and accumulate in the blood of ovariectomized females. Moreover, these antigens are female-specific, occur only in adult, vitellogenic females and, as indicated by their dense bands of precipitation in Oudin tubes, comprise a major portion of the yolk protein complement. In view of these findings, the antigens responsible for the formation of precipitin bands A and B are referred to here as vitellogenins. This term was applied by Pan, Bell and Telfer (1969) to designate a functionally discrete group of insect blood proteins which are female-specific and which are selectively sequestered by the oocytes.

Figure 2 illustrates the results of using the Oudin test to measure the concentration of vitellogenins A and B in *B. fumigata* blood or serially diluted yolk fluid (in this case the antiserum was previously absorbed with male blood in order to remove antibodies homologous to antigens C and D). It has been shown by Oudin that a linear relationship exists between the logarithm of the antigen concentration and the value K (distance of migration [mm] by the leading edge of a precipitin band divided by the square root of time [min]). With regard to the above relationship, the distance of migration of the precipitin bands in tubes containing serial dilutions of yolk fluid were measured and these distances were divided by the square root of time; the resulting K values were plotted against the concentration of yolk fluid (Fig. 3). The rates of migration of the bands in tubes layered with blood from 15-day females, pregnant females and 16-day ovariectomized females were also measured and the concentration of the vitellogenins in these solutions, relative to their concentrations in the yolk fluid, was ascertained by referring to the standard curve shown in Figure 3. Table I shows the concentration of the vitellogenins in the solutions tested.

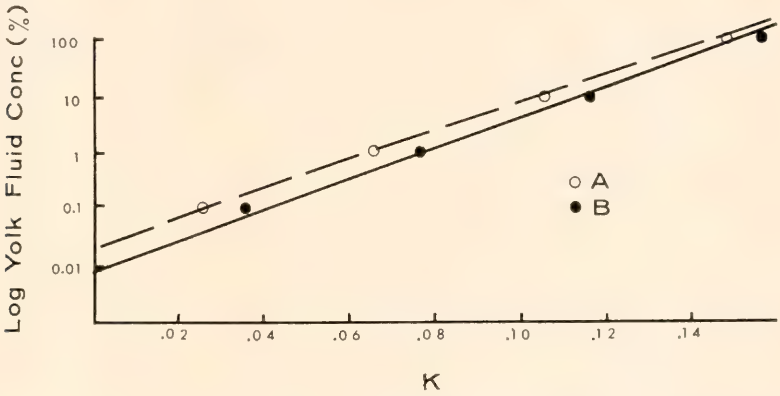

Figure 3. Logarithm of yolk fluid concentration plotted against K values [distance of migration of precipitin bands (in mm) divided by the square root of the time interval (in minutes)].

In the studies presented in this paper, the relative concentrations of the vitellogenins were measured in the blood of females during the reproductive cycle in order to ascertain the relationship between the yolk precursor secretion cycle and other reproductive processes.

5. *Colleterial gland glucoside activity*

The spectrophotometric glucoside assay of Willis and Brunet (1966) was employed to measure the secretory activity of colleterial glands. The left colleterial glands were excised and homogenized in 2 ml of saturated ammonium sulfate. 0.2 ml of the supernatant was mixed with 2.8 ml of distilled water and the solutions were read against appropriate blanks in a Carey recording spectrophotometer.

Colleterial gland extracts contained the glucoside (G1-A) which was previously characterized by paper chromatography (Stay and Roth, 1962) and was found to have an absorption spectrum at 2770 Å. A second component, with a peak of 2850 Å, was observed in glands excised from newly emerged females (Days 0 to 8). Glucoside units (a change in O.D. of 0.001) were measured in colleterial glands from females during the reproductive cycle.

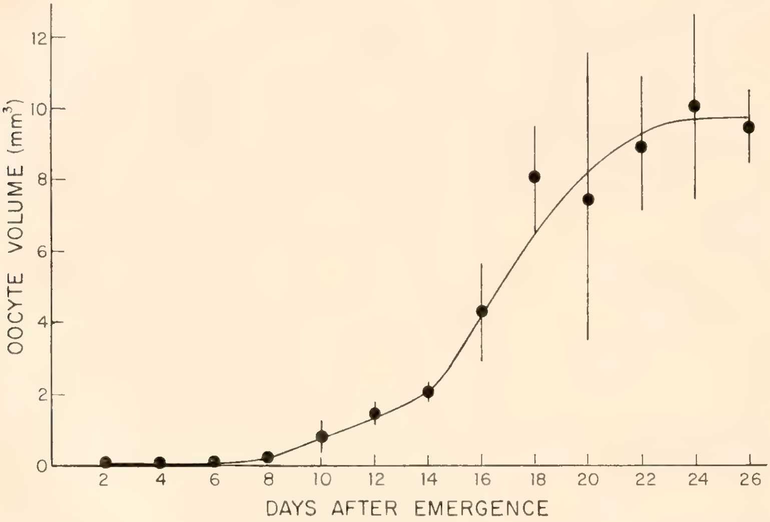

FIGURE 4. Changes in oocyte volume during the first preoviposition period. Vertical lines are standard deviations. Each point represents the average of 10 to 20 females.

The width of the left colleterial gland tubules was also used as an indication of gland activity. In newly emerged females the gland tubules are white and have an average width of 0.1 mm, while in vitellogenic females the tubules are bluish green and have an average width of 0.6 mm.

RESULTS

1. *Yolk deposition*

The onset of yolk deposition occurs on about day 5 after ecdysis; the basal oocytes are characterized by detectable quantities of extractable vitellogenin, an increase in volume and the appearance of refractile yolk spheres in the oocyte cortex. Rapid deposition of vitellogenic blood proteins occurs during days 14 to 22, followed by a period of less intense deposition and terminating completely by day 26. Oocytes with an average volume of 10 mm^3 were ovulated between days 23 and 30 (average of 26) after emergence (Fig. 4). Employing essentially similar culture conditions and techniques as in the present study, Roth and Stay (1962) reported that in their colonies the mated females oviposited between days 26 and 41 (average of 32.4). This apparent reduction in the period required for oocyte maturation in insects cultured over a long period of time has also been observed in *Schistocerca gregaria* (L. Hill, University of Sheffield, personal communication).

Female *B. fumigata* are normally receptive to courting males between days 10 and 30 after emergence. In females which fail to mate, the oocytes often fail to develop or may be resorbed after partial development; moreover, nearly all virgins (including those which do oviposit) lack the synchronous oocyte growth observed in mated females. Virgin females which oviposit do so slightly later than mated females (Roth and Stay, 1962).

2. *Vitellogenin secretion*

The blood of females on day 4 or 5 after emergence contains slight, but unmeasurable quantities of vitellogenin (less than 0.001% relative to the yolk fluid standard), suggesting that these yolk precursors are present, but only in trace amounts. Significant levels of both vitellogenins appear in the blood of most females on day 7 or 8. Antigen B is detected about 24 hours prior to the appearance of antigen A, and throughout the cycle antigen B occurs in higher concentra-

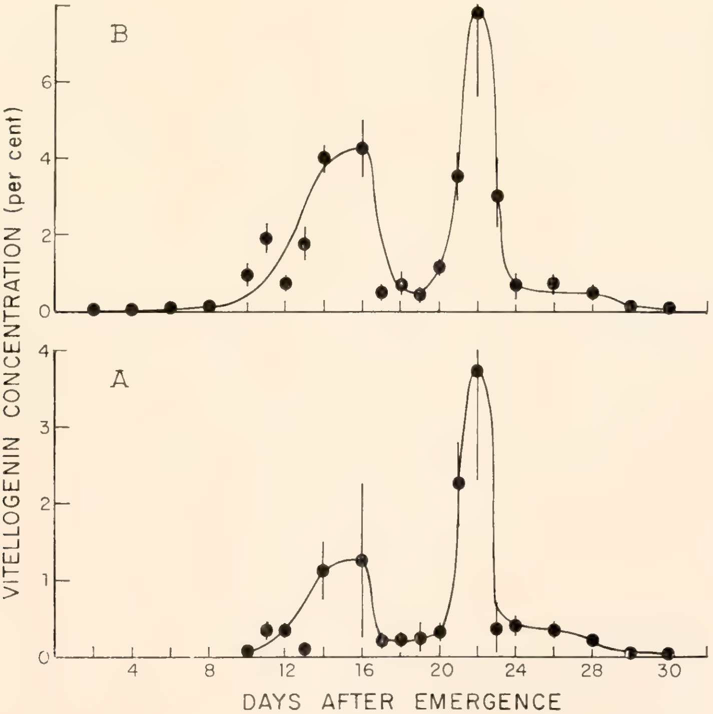

FIGURE 5. Changes in blood vitellogenin concentration during the first preoviposition period. Vertical lines are standard deviations. Each point represents an average of 10 to 15 females.

tion than antigen A (relative to their concentration in the yolk fluid standard). From the time of their first appearance in female blood the vitellogenins increase synchronously to a peak on day 16 (Fig. 5), drop to a low level by day 18, increase to a second peak at day 22 and then decrease in concentration until ovulation. Finally, in most females the vitellogenins disappear entirely from the blood during pregnancy; indeed, by the tenth day of pregnancy the vitellogenins could not be detected in 80% of the females tested.

The trough in the vitellogenin curve between days 17 and 20 corresponds to the period of most intense yolk investment. During this period the oocytes increase in volume by 4.00 mm^3, an increment which is greater than that of the previous or the succeeding 4 day period. Secondly from days 20 to 24 the rate of increase in oocyte volume is substantially less than during any other period in the vitellogenic cycle; this hiatus in yolk deposition may account for the observed replenishment of blood vitellogenin which reaches a peak on about day 22. Resorption of some basal oocytes which commonly occurs even in mated females towards the end of the vitellogenic period, may also contribute to the second peak in vitellogenin concentration.

Measurements were made of the vitellogenin concentration in the blood of ovariectomized females. The accumulation of the protein yolk precursors in castrated females (which reaches a concentration of 4.0% [A], and 10.9% [B] at day 16) gives credence to the role of these proteins in yolk deposition; moreover, the observation that the vitellogenin concentration of ovariectomized females declines to 1.4% [A], and 4.6% [B] at the time when normal females are initiating egg incubation suggests that the observed decreasing concentrations of blood vitellogenin towards the end of vitellogenesis is due to processes in addition to the removal of these proteins from the blood by yolk-forming oocytes.

3. *Pheromone production*

On the basis of the filter paper assay it was reported that 90% of normal virgin females initiated pheromone production between 10 and 30 days after the imaginal molt (Barth, 1961, 1962). However, it was also noted that females maintained with males from emergence occasionally mated as early as day 4 (Barth, 1962). This observation together with other studies demonstrating the importance of the sex pheromone for the release of male courtship behavior (Barth, 1964) led to the conclusion that the pheromone is very likely present on the body surface of the female at an earlier age than that at which it is detectable on filter paper. For this reason the pheromone assay employing the female as described above was extensively employed in these studies. When introduced into a chamber of males, the test females usually showed the immobilization reaction (flattening against the substratum and withdrawing the antennae laterally beneath the pronotal shield—Barth, 1964), or less commonly attempted to escape by hiding under a nearby object. Such females were invariably sexually unreceptive and behaviorally were essentially as inert as filter papers, hence any stimuli presented to the males are highly likely to have been chemical alone.

Table II indicates that by day 4 a sufficient quantity of pheromone was present on the cuticle of 5% of the females tested to elicit a courting response by adult males. On day 10, 94% of the females placed in male cages elicited the male court-

Table II

*Initiation of pheromone production**

	Days after emergence											
	1	2	3	4	5	6	7	8	9	10	11	12
% of females with detectable cuticular pheromone	0	0	0	5	17	32	52	74	84	94	100	100

* Represents data from 65 females.

ing response. It is clear that by means of this assay procedure, data can be obtained which bring the initiation of pheromone secretion into line with the initiation of other reproductive processes at the onset of the reproductive cycle. Barth (1970) reported that the pheromone is a product of some portion of the female genital tract other than the colleterial glands; thus according to our current hypothesis, the pheromone is secreted through the genital opening and is selectively adsorbed onto the surface of the cuticle. It is also adsorbed onto the surface of other objects in the vicinity (*i.e.,* filter papers). Whether such objects possess sufficient pheromone activity to stimulate the males depends upon the rate of pheromone output by the female relative to the evaporation and/or breakdown rate of the pheromone. Output rates seem to vary considerably and may be characteristic of individual females (Barth, 1962).

As reported by Barth (1968) and as shown in Table III, pheromone production ceases within a few days after mating. In some individuals it may cease as early as the first day and in no case has it been observed to continue beyond the third day following mating. By contrast virgin females continue to produce the pheromone until they ovulate; moreover, if they fail to ovulate on schedule (Roth and Stay, 1962), they may continue to produce sex pheromone for several weeks.

A related event of importance which occurs early in the reproductive cycle of the female is the onset of sexual receptivity. Previous work on sexual receptivity suggests that in contrast to the other four processes under discussion, it is not regu-

Table III

*Cessation of pheromone production by females after mating**

Number of females tested	Age (in days after mating) when first tested	% females with positive pheromone
66	0†	100
4	1	0
31	2	0
16	3	33‡
15	4	0

* Filter paper assay used in all tests. Females mated at 10–16 days of age one day after demonstrating pheromone production by means of the filter paper assay.

† Indicates the day on which females were mated.

‡ These females all ceased pheromone production on day 4 after mating.

lated directly by the corpora allata (Roth and Barth, 1964; Barth, 1968; Engelmann and Barth, 1968). The results of the female pheromone assay above indicate that the female herself is not receptive at the time when sex pheromone on the surface of her cuticle makes her attractive to males. In view of our interest in the sequence of reproductive events we continued to test a small number of active females daily for signs of receptivity. All six animals so tested showed antennation and mounting of displaying males within the two minute test period on the second day following appearance of pheromone on the cuticle. Thus females appear to reach a high level of sexual receptivity about 2 days after the onset of pheromone secretion. It may be noted that this is likely to be several days before the average onset date for pheromone secretion as determined by the filter paper assay. Herein lies the probable explanation for the accounts in the literature of mating in *B. fumigata* females prior to the onset of pheromone production (Barth, 1962; Roth and Stay, 1962).

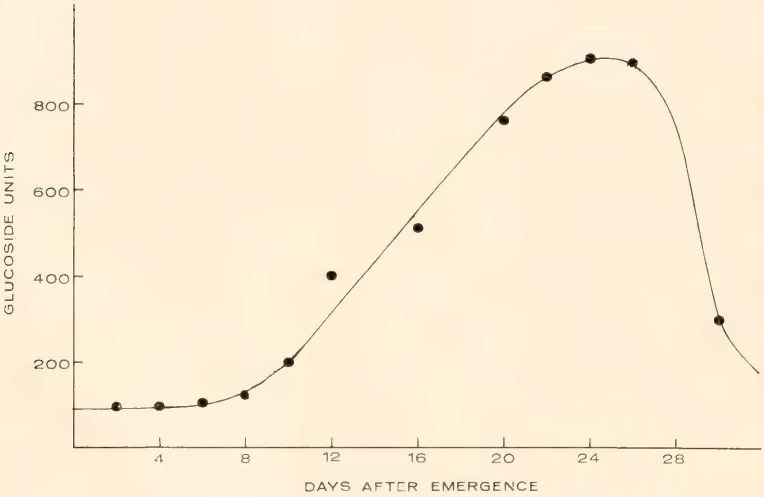

FIGURE 6. Changes in left colleterial gland glucoside concentration during the first preoviposition period. Each point represents the average glucoside units measured for the pooled glands of 5 females.

4. Colleterial gland activity

The left colleterial gland of *B. fumigata* secretes the structural protein of which the ootheca is composed and a glucoside of the phenolic tanning agent (Stay and Roth, 1962). Synthesis and storage of the protein is indicated by a bluish green color and increase in width of the gland tubules. The appearance of color and increase in colleterial gland size was observed to occur between days 4 and 6. In addition to the glucoside with an absorption peak of 2770 Å, the glands contained an unknown substance with an absorption peak of 2850 Å; this unknown was present in the left colleterial gland extracts (in saturated ammonium sulfate) until between days 6 and 8 at which time the unknown was replaced by the glucoside.

The presence of the left colleterial gland glucoside of *B. fumigata* (G1-A), identified chromatographically by Stay and Roth (1962), was first detected between days 6 and 8 after adult ecdysis. The units of activity increased linearly (Fig. 6) in mated females until a leveling off point occurred on about day 24. In virgin females the individual variation was very large owing to the low concentration of glucoside observed in many of these animals after day 15.

Following ovulation and oviposition there was a marked decline in glucoside activity, but some glucoside is apparently retained by the colleterial glands throughout the pregnancy period. It is interesting to note that the average period of pregnancy (53.1 days) observed in these studies is substantially shorter than the average pregnancy period of 76.2 days recorded by Roth and Stay (1962); these findings provide further evidence for the acceleration of reproductive processes in insects cultured for a number of generations.

DISCUSSION

Four reproductive processes in the cockroach *B. fumigata* are known to be influenced by juvenile hormone (Bell and Barth, 1970). These processes appear to be initiated simultaneously in females on about the fifth day after adult emergence. An attempt was made in the present study to determine whether the initiation of any one process consistently preceded any other, but this was shown not to be the case.

A number of analogies between the reproductive cycle of *B. fumigata* and those of other cockroach species are revealed by the study reported here. The crucial difference between the two major cockroach groups is that the cycle of the family Blaberidae includes a period of pregnancy which is absent from the cycle of the superfamily Blattoidea and the family Blattellidae of the superfamily Blaberoidea (classification according to McKittrick, 1964) (see Fig. 1). This difference brings about obvious changes in the schedule of yolk formation, vitellogenin secretion, colleterial gland function and pheromone secretion; in *P. americana*, for example, the cycle of vitellogenin secretion and sequestration by the oocytes is continuous owing to the absence of a pregnancy period (Bell, 1969b). Among several consequences of a continuous cycle as exemplified by *P. americana* is the elimination of a peak in blood vitellogenin found in many *B. fumigata* females at the termination of yolk formation. In *P. americana* the second (penultimate) set of oocytes, which begins development prior to the termination of development in the first (basal) set, accumulates any vitellogenins which are not sequestered by the basal oocytes. On the other hand in *B. fumigata* there is a small quantity of blood vitellogenin which is not utilized and which remains in the blood during the first 10 to 20 days of pregnancy.

Another consequence of having continuous reproductive cycles and probably continuous juvenile hormone secretion, is that vitellogenin secretion and yolk deposition do not occur periodically, but seem to be maintained constantly throughout the reproductive life of the female (Bell, 1969b). Colleterial gland activity, on the other hand, proceeds somewhat differently for reasons of cyclic demand; during ootheca formation in *P. americana* the colleterial gland glucoside activity is reduced by 75%, but the supply is replenished within 2 days after ootheca formation (Willis and Brunet, 1966).

In *B. fumigata* the onset of pregnancy brings about a concomitant termination of vitellogenin secretion and yolk deposition; the same is true in *Leucophaea maderae,* another oviviparous species (Engelmann and Penney, 1966). The colleterial gland glucoside activity is reduced by 78% during ootheca formation in *B. fumigata,* but it is not until parturition that the gland begins to fill again; parturition is also the point in the reproductive cycle at which yolk deposition and vitellogenin secretion are again initiated.

Pheromone activity is first associated with the cuticle of day 4 females and may ultimately be detected on filter papers in beakers housing day 10 or older females. It is not known whether pheromone transfer to the filter papers is due to an increased pheromone output by the females or to a change in the structure of the pheromone molecule. The absence of a precise correlation between the pheromone secretion schedule and the schedule of female receptivity, as observed in our study, substantiates earlier work on *B. fumigata, Nauphoeta cinerea* and *L. maderae* (Roth and Barth, 1964; Barth, 1968), suggestive of different control mechanisms for the two processes.

The properties and functions of the two *B. fumigata* vitellogenic blood proteins suggest that they are analogous to similar proteins observed in *L. maderae* (Dejmal and Brookes, 1968; Engelmann and Penney, 1966; Engelmann, 1969), and *P. americana* (Bell, 1969b, 1970). In all three cases the vitellogenins require a high ionic strength for solubility and are selectively incorporated from the blood by yolk-forming oocytes. In the three species mentioned above, the two primary yolk components can be extracted only if the medium contains a salt concentration of at least 0.4 *M* NaCl. Whereas the vitellogenins are the primary yolk constituents, other protein components do exist in cockroach yolk as demonstrated electrophoretically or immunochemically by Adiyodi and Nayar (1967), Bell (1970), Nielsen and Mills (1968) and Scheurer (1969b). These proteins, however, are present in relatively small amounts and do not exceed their relative concentration in blood; hence they are not selectively sequestered by the oocytes.

With regard to the hormonal effect, it should be noted that even though the onset of juvenile hormone secretion is thought to occur at about the same time as the initiation of the various reproductive processes, it is impossible to determine at this time whether the hormone acts directly upon the ovaries (yolk deposition), fat body (yolk precursor synthesis), colleterial glands and pheromone gland; indeed, until the molecular effects of the hormone are further elucidated we will not know the precise relationship between the effector sites of juvenile hormone and the processes which we have studied. Specific effects of the hormone will be the topic of subsequent papers in this series.

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SUMMARY

1. Four reproductive processes (vitellogenin secretion, yolk deposition, colleterial gland activity and pheromone production) are initiated on about the fifth day

after adult ecdysis. There is no apparent sequence of initiation of these processes except that vitellogenin secretion either precedes or occurs concomitantly with the initiation of yolk deposition.

2. Two blood proteins, which are female-specific yolk precursors (vitellogenins A and B), reach a concentration peak on day 16 and then decrease in response to rapid yolk deposition. A second peak on day 22 is thought to represent yolk precursors which were released to the blood as a result of the resorption of some basal oocytes. The vitellogenins are either absent or occur in trace quantities in the blood of pregnant females.

3. Sex pheromone is detectable on the surface of the cuticle by day 10 in 94% of the females tested. Following mating pheromone production is drastically curtailed.

4. Glucoside activity in colleterial glands increases almost linearly from day 8 to 24. After ootheca formation, which involves the utilization of colleterial gland secretion, there is a 78% reduction in glucoside activity.

5. The sequence of events in the reproductive cycle of females of *B. fumigata* is compared with that of other cockroaches.

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EXPERIMENTAL STUDIES ON THE ENDOCRINOLOGY AND REPRODUCTIVE BIOLOGY OF THE VIVIPAROUS POLYCHAETE ANNELID, *NEREIS LIMNICOLA* JOHNSON [1]

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The reproductive biology of relatively few Northeastern Pacific nereids has been described: *Nereis limnicola* (as *Neanthes lighti*) (Smith, 1950), *N. vexillosa* (Johnson, 1943), *N. grubei* (Reish, 1954; Schroeder, 1968), *N. caudata* (Reish, 1957), and *Micronereis nanaimoensis* (Berkeley and Berkeley, 1953). Gould and Schroeder (1969), Schroeder (1967, 1968), and Baskin (1970) have reported on experimental studies related to endocrine control of reproduction and development.

The reproductive endocrinology of *N. limnicola* is of interest for a number of reasons. This viviparous species has a close relationship with, and is virtually indistinguishable from, the oviparous *Nereis diversicolor* (Smith, 1958), the subject of previous studies on the hormonal control of reproduction (Durchon, 1952; Clark and Ruston, 1963; Durchon and Boilly, 1964; Durchon and Dhainaut, 1964; Durchon and Dhainaut-Courtois, 1964; Durchon and Porchet, 1970). The embryology and reproductive biology of *N. limnicola* has been studied by Smith (1950). These viviparous worms are self-fertilizing hermaphrodites; fertilization occurs in the coelom, where development proceeds until the larvae are 4–5 mm in length and have approximately 20–30 pairs of parapodia. Parturition occurs by ruptures of the body wall of the adult. This reproductive pattern is unique among nereids (Smith, 1958) and raises the possibility of endocrine regulation of viviparity.

Both somatic and gametic maturation are known to be hormonally controlled in nereids (see reviews by Clark, 1965, 1969; Hauenschild, 1965; Durchon, 1967). The primary oöcytes are shed from a proliferative epithelium and grow within the coelomic cavity (Dales, 1950; Durchon, 1952). Once the oöcytes have reached a critical diameter, removal of the brain (supraesophageal ganglion) may result in a phase of rapid oöcyte growth, although this response is variable in different species (Durchon, 1952, 1956; Clark and Ruston, 1963; Hauenschild, 1965, 1966; Dhainaut and Porchet, 1967; Malecha, 1967; Schroeder, 1968). Typically, somatic and gametic maturation coincide, whether somatic maturation consists of metamorphosis into the epitokous heteronereid form, or in the less spectacular modifications characteristic of atokous species, and it is thought that both processes are controlled by the declining titer of a single hormone of presumed cerebral origin (Golding, 1967c). However, several observations by Smith (1950), as well as by the present authors indicate that the temporal relationships of the gametic and somatic aspects of maturation in *N. limnicola* differ from the typical nereid pattern.

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The source of the maturation-inhibiting hormone in nereids has been presumed to be neurosecretory cells of the supraesophageal ganglion (see Gabe, 1966; Golding, 1967b; Dhainaut-Courtois, 1968b, for reviews). More recently, however, it has been suggested that the infracerebral gland, a possible neuroendocrine complex on the ventral surface of the cerebral ganglion, might be the source of this factor (Dhainaut-Courtois, 1968a; Golding, Baskin and Bern, 1968). This complex is composed of an epithelium that contains two principal cell types: (1) the prominent *a* cells, which in transverse section give the gland the configuration of a columnar epithelium and whose cytoplasm is devoid of electron-dense granules; (2) the relatively scarce *b* cells, which are irregular in shape and which contain electron-dense cytoplasmic granules. Neurosecretory axons originating from within the brain pass through the fibrous brain sheath and are found amongst the cells of this epithelium. The endings of other neurosecretory axons are found adjacent to the inner surface of the fibrous sheath that encapsulates the brain and separates it from the epithelium.

Since previous work on nereid reproductive endocrinology had been carried out on oviparous species, it was of special interest to determine if endocrine control of gamete maturation in *N. limnicola,* a viviparous species, conforms to the general nereid pattern. Golding, Baskin and Bern (1968) observed that the infracerebral gland epithelium of *N. limnicola* was thicker than that of other nereids, and speculated that the unusually well-developed infracerebral gland of *N. limnicola* might be related to the viviparous reproductive pattern of this species.

The present paper reports on several experiments related to the endocrine control of reproductive maturation in *N. limnicola.* Furthermore, possible endocrine influence on development of larvae within the coelom was studied. Finally, parts of a severed brain were implanted into decerebrate worms in order to examine the possible relationship of the infracerebral gland to production of the maturation-inhibiting hormone.

Materials and Methods

Specimens of *N. limnicola* were collected at Lake Merced in San Francisco, California. They were gradually adapted to 25% sea water at 14° C in the laboratory for several days before being examined for gametes. This procedure was followed since worms adapted to lower salinities usually did not survive anesthesia. The choice of 25% sea water was made because this salinity falls within the range in which *N. limnicola* regulates the osmotic concentration of its coelomic fluid at levels comparable to that when the worms are adapted to Lake Merced water, which has a salinity of 0.5% sea water and is considered to be fresh (Oglesby, 1965). Each worm was removed from the water and blotted on filter paper. All coelomic fluid samples were of approximately identical volume, and were taken using a fine-tipped capillary tube and examined under a coverslip in a drop of mineral oil. All measurements were taken at 100 × magnification, and oöcyte diameters were measured with an ocular micrometer. As several size classes of oöcytes were usually present in an individual, only the diameters of the largest oöcytes found in the sample were utilized, following the procedure used by Schroeder (1968). Terminology for the developmental stages and larvae follows Smith (1950).

The quantity of motile sperm present in each worm was evaluated on an ordinal scale of 0 to 5 as follows:

0 no motile sperm present in entire sample.
1 less than 10 motile sperm present in entire sample.
2 less than 10 motile sperm present in an average field.
3 11–20 motile sperm present in an average field.
4 21–50 motile sperm present in an average field.
5 more than 50 motile sperm present in an average field.

Because the sampling procedure usually required several days, the worms were held in a refrigerator (6° C) until all specimens used in an experiment had been examined.

All of the worms used in each experiment, including the intact controls, were anesthetized in 5% ethanol in 25% sea water. Decerebration was carried out as described by Golding (1967a). In the case of decerebrate worms which received an implanted brain, the excised brain was inserted into the coelom by pushing it posteriorly through the wound resulting from the brain removal operation. In experiments in which portions of the brain were implanted, the excised brain was placed on filter paper under a dissecting microscope and the cut made with fine-tipped scissors. The desired portion of the brain was then implanted into the coelom as described above. Single parapodia were removed from each worm to identify individuals. The jaws of worms with intact brains were snipped off at the base to prevent cannibalism. All wounds sealed themselves by contractions of surrounding tissues. After the operations, the worms were placed in 25% sea water which had been filtered, boiled, and cooled, and to which 140 mg/liter streptomycin sulfate (Upjohn) had been added. The worms were maintained in plastic refrigerator dishes containing several layers of glass tubes (as described by Golding, 1967a) at a constant 18° C, and were not fed during the experiments. In all cases, the worms appeared healthy and vigorous up to the completion of the experiments.

The results were analyzed statistically using the non-parametric Fisher exact probability, Chi-square, and Mann-Whitney *U* tests from Siegel (1956).

The brains used for histology were fixed in Helly's fluid, sectioned at 5 μ in Paraplast, and stained with paraldehyde fuchsin (Clark, 1955).

RESULTS

The relationship of somatic and gametic maturation

Several somatic changes that are associated with reproductive maturation of other nereids appear to be delayed in *N. limnicola*. The extensive histolysis of body musculature, which characteristically accompanies nereid gametic maturation (Defretin, 1949), does not coincide with sperm and oöcyte maturation in this species. Our observations indicate that muscular histolysis in *N. limnicola* occurs after the gametes have matured and is correlated with the presence of advanced larval stages in the coelom. Furthermore, as reported by Smith (1950), the coelomocytes are abundant in the coelomic fluid of worms with mature oöcytes, and the coelomocyte concentration does not decline until after cleavage stages are present in the coelom.

Another aspect of somatic maturation that is associated with the reproductive maturity of nereids is the loss of regenerative ability. Adult worms regenerate a pygidium but only rarely regenerate lost segments (Golding, 1967c). In order to determine if this aspect of maturation is delayed in *N. limnicola,* six specimens containing mature oöcytes (130–160 μ in diameter), but no larvae, were kept for three weeks after removal of about one-half of their posterior segments. Each worm regenerated a pygidium but did not regenerate any segments. These results indicate that regenerative growth does not occur during the later stages of gamete maturation of *N. limnicola.*

TABLE I

The effect of decerebration on gamete maturation

Group	Experiment 1*				Experiment 2**						
	Number of worms		Worms with larvae		Number of worms		Worms with larvae		Worms with motile sperm		Motile sperm average score
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	
Decerebrate	15	14	0	12	18	18	0	16	3	17	3.2
Intact	15	12	0	6	18	17	0	2	2	11	2.1
Implant	15	14	0	1	18	18	0	4	1	11	1.6

* 19 days.
** 3 weeks.

Effect of decerebration on gamete maturation

Experiment 1. Forty-five animals were separated into three groups of equal size. A sample of coelomic fluid was obtained from each animal and both the maximum and mean oöcyte diameter was determined for each worm. The absence of sperm was confirmed for each specimen. The three groups were subjected to the following treatments, respectively: *Intact,* brain left *in situ; Decerebrate,* brain removed; *Implant,* brain removed and implanted into the coelom.

The groups were maintained under identical conditions. After 19 days, further samples of coelomic contents were obtained, and the presence or absence of larvae was noted. The results, summarized in Table I, showed that a significantly greater number of *Decerebrate* worms contained larvae than did the *Implant* group (Fisher, $P < 0.005$). The *Intact* and *Implant* groups also differed in this respect (Fisher, $P < 0.05$), but the difference between the *Decerebrate* and *Intact* groups was not significant.

Experiment 2. In a second experiment, the coelomic contents of 54 worms were examined and three groups of equal size were established as in experiment 1: *Intact, Decerebrate,* and *Implant.* Worms at various stages of maturity were apportioned equally among the three groups. Each group was maintained in a separate container under identical conditions. After eight days, a small sample of coelomic fluid was taken from each worm and examined for sperm. Three weeks after the initial sample, each worm was re-examined for oöcytes, sperm and larvae.

The results of experiment 2 are shown in Table I. Clearly, significantly more specimens of the *Decerebrate* group contained larvae than either the *Intact* or *Implant* groups (Fisher, $P < 0.001$). There was no difference between the *Implant* and *Intact* groups in this respect. Hence, the precocious appearance of coelomic larvae, which followed brain removal, did not occur if a brain had been implanted into the coelom of the decerebrate worm.

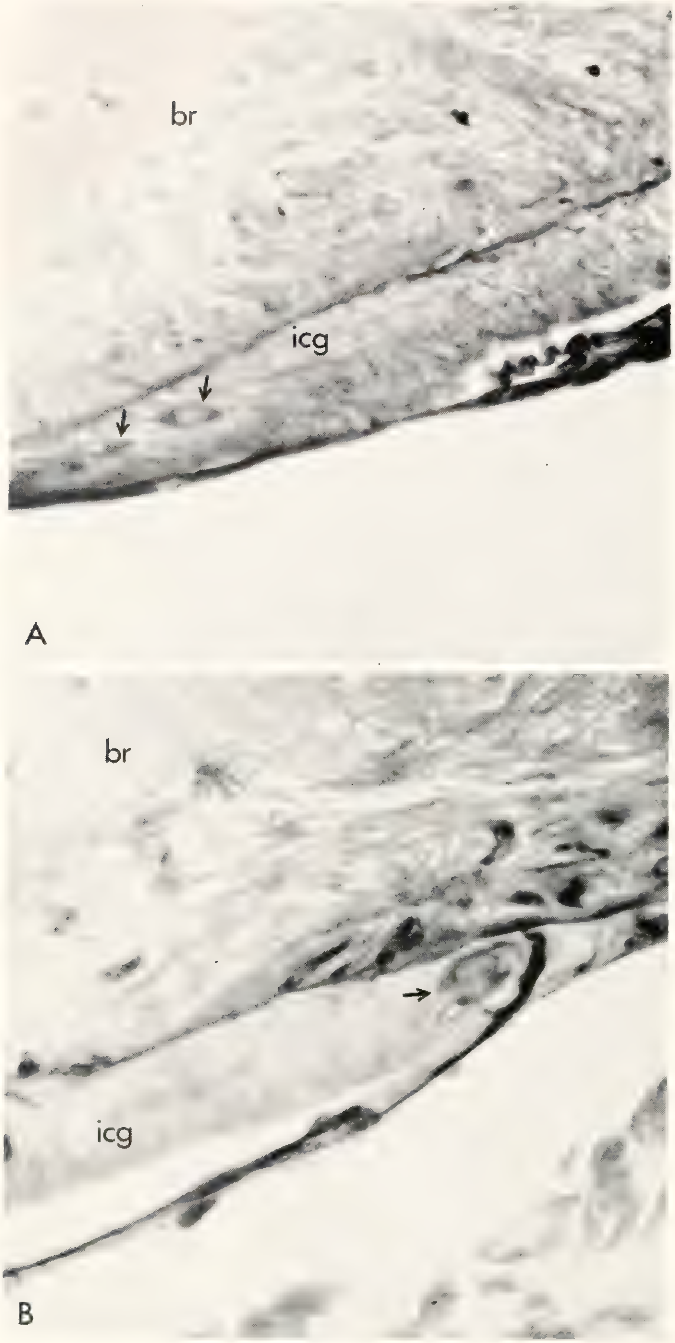
There was no difference among the three groups with respect to the presence of motile sperm after eight days. After three weeks, however, a greater proportion of individuals in the *Decerebrate* group had motile sperm as compared to the *Intact* and *Implant* groups (Fisher, $P < 0.05$). The average scores for quantity of sperm, as shown in Table I, indicate that the *Decerebrate* worms produced more sperm compared to the *Implant* worms (Mann-Whitney, $P < 0.01$). Although the *Decerebrate* group also produced more sperm than did the *Intact* specimens, the scores were not significantly different. Nevertheless, the only strictly comparable groups are the *Decerebrate* and the *Implant*, and the results indicate that brain removal results in the production of abnormally more sperm as well as precocious sperm maturation.

Histology of the implanted brains

At the conclusion of experiment 2, the brain was recovered from the coelom of each worm in the *Implant* group. In each case, the ganglion, which was implanted at the level of the prostomium, was located in the posterior region of the body, and was floating unattached in the coelomic fluid. The brains were intact and showed no pronounced allometric changes, and the eyes were in a relatively normal position. Histological examination revealed that the infracerebral gland epithelium was normal in appearance. A comparison of the implanted and *in situ* brains revealed only subtle differences in the appearance of this epithelium (Fig. 1). There was the impression that, in the case of implanted brains, the *b* cells were more numerous, since they seemed to stain slightly more intensely. However, the differences were not evaluated quantitatively. The *a* cells did not exhibit unusual hypertrophy or atrophy, nor did they show significant storage or depletion of cytoplasmic inclusions, as compared with the *in situ* brains, although the *a* cells of implanted brains sometimes showed a slight peripheral deposition of granular material (Fig. 1A). Further, the implanted brains showed no unusual difference in the stainability of the cells and fiber tracts within the cerebral ganglion.

Effect of decerebration on viviparous development

Three groups of 13 worms each were established as described in the previous experiments: *Decerebrate*, *Intact* and *Implant*. A parapodium was removed from each worm for purposes of identifying individuals. Initially, all worms had developmental stages present in the coelom; the oldest stages present varied from gastrulae to second-cirrus larvae. However, each group had about the same distribu-


br
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A
br
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FIGURE 1.

tion of developmental stages. Following the operations, all of the worms were maintained in the same dish containing aerated 25% sea water at 18° C.

After 21 days, the worms were re-examined for larvae; the results are shown in Table II. Coelomic larvae were present in only 45% of the *Intact* group and 66% of the *Implant* group, whereas all survivors of the *Decerebrate* group contained larvae. The *Decerebrate* group thus had a greater proportion of individuals which still had coelomic larvae as compared to the *Intact* (Fisher, $P < 0.01$) and *Implant* (Fisher, $P < 0.05$) groups; there was no significant difference between the latter two groups. However, of those worms which contained larvae in each group, all had larvae and juveniles up to 30 setigers. No worms underwent parturition.

TABLE II

The effect of decerebration on viviparous development

Group	Number of worms		Stages present at conclusion*	Number of worms*	
	Initial	Final*		With larvae	Without larvae
Decerebrate	13	11	19–30 setigers	11	0
Intact	13	11	26–29 setigers	5	6
Implant	13	12	24–30 setigers	8	4

* After three weeks.

Effect of implantation of parts of brains on gamete maturation in decerebrate worms

After initially sampling the coelomic fluid contents of 76 worms, the following groups were established:

Decerebrate Brain removed (15 worms),
Intact Implant Brain excised and implanted into the coelom (16 worms),
Dorsal Implant Dorsal part of the excised brain implanted (15 worms),
Ventral Implant Ventral part of excised brain implanted (15 worms),
Dorsal and Ventral Implant Brain cut and both the dorsal and ventral parts implanted (15 worms).

Initially, all worms contained oöcytes, and the maximum oöcyte diameters varied from 50 μ to 139 μ, but in each group the distribution of worms at various stages of maturity was similar. Sperm were not present in any of these worms. The brain was removed from each worm and, where appropriate, cut in a horizontal plane in order to separate the ventral region, on which is located the infracerebral gland epithelium, from the dorsal part, in which are located many neurosecretory cells. The desired portions were then implanted into the coelom.

FIGURE 1. A. This infracerebral gland epithelium is from a brain recovered after three weeks of implantation into a decerebrate worm. B. This infracerebral gland epithelium is from a normal *in situ* brain; *br*, brain; *icg*, infracerebral gland epithelium *a* cells. The arrows point to *b* cells. (Both A and B: Helly, paraldehyde fuchsin, 500 ×.)

TABLE III

The effect of implanting parts of a brain on gamete maturation in decerebrate worms

Group	Number of worms		Coelomic fluid contents*		
	Initial	Final*	Oöcytes	Larvae	Neither
Decerebrate	15	15	0	11	4
Intact implant	16	16	14	1	1
Dorsal implant	15	12	4	6	2
Ventral implant	15	14	3	9	2
Dorsal and ventral implant	15	15	6	9	0

* After 19 days.

After 19 days, each worm was examined for oöcytes and larvae. The results are summarized in Table III. The *Decerebrate* group had significantly more worms with larvae than the *Intact Implant* group (Chi-square, $P < 0.001$). The *Dorsal, Ventral,* and *Dorsal* and *Ventral Implant* groups were similar in having several worms containing larvae, and thus resembled the *Decerebrate* group in this respect; however, they differed from the *Decerebrate* group in that each of the former had several worms containing oöcytes, but no larvae. The *Intact Implant* group had significantly fewer worms with larvae than did those of the *Dorsal Implant* and *Ventral Implant* groups (Chi-square, $P < 0.01$).

It is conceivable that differences could exist between the groups as to the developmental progress of individual worms, since all stages from cleavage to second-cirrus larvae are included in the category, "larvae." This possibility was examined by considering only the worms containing either oöcytes or larvae (or both). Although larvae of several developmental stages (as well as oöcytes) were often present in an individual, the most advanced stage present was considered an index of developmental progress. The most advanced stages present in individual worms of each group were tabulated and expressed as a percentage indicating the relative proportion of those individuals within each group (Table IV). The data suggest that the decerebrate worms were more advanced with respect to the developmental progress of their coelomic larvae as compared to the other groups, and this impression is strengthened if the groups are compared as to the proportion of each group that reached selected developmental stages, as shown in Figure 2.

TABLE IV

The percentage of worms containing oöcytes or larvae at selected developmental stages for each group in Table III

Group	Oöcytes	Cleavages	Gastrulae	Trochophore	3-segment larvae	Tentacle-bud larvae	2nd-cirrus larvae
Decerebrate	0	0	10	27	18	27	18
Intact implant	93	7	0	0	0	0	0
Dorsal implant	40	0	0	20	10	30	0
Ventral implant	26	8	33	8	17	0	8
Dorsal and ventral implant	40	0	0	13	27	20	0

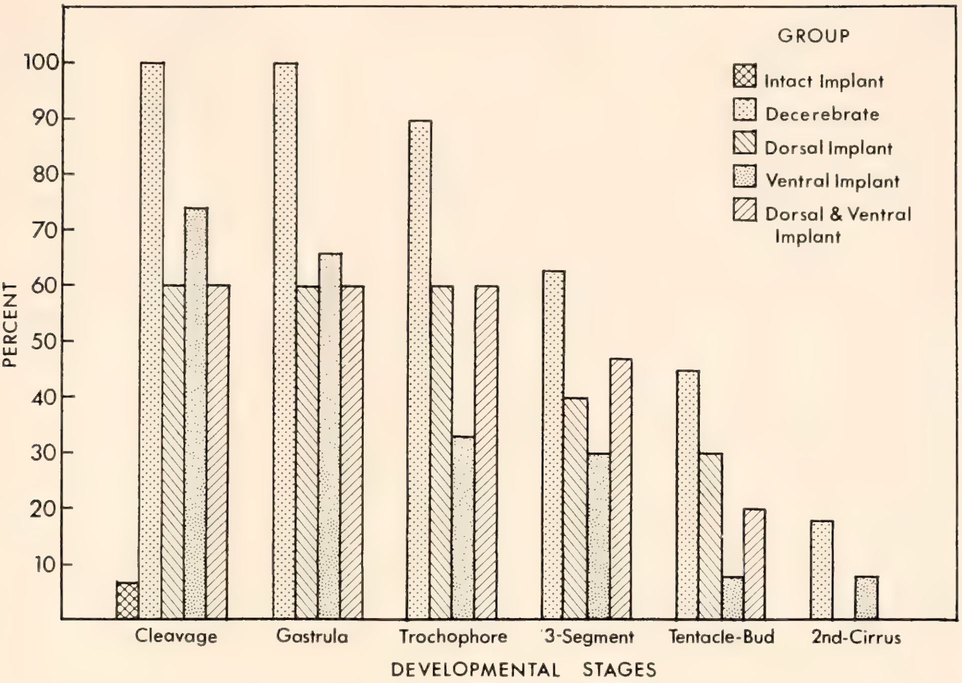

FIGURE 2. This figure compares the developmental progress of the groups in the brain-cutting experiment. The horizontal axis represents larval stages in sequence of development. The vertical axis represents the percentage of worms, in each group, containing larvae which had advanced to the stages indicated. The percentages are based on the worms that contained oöcytes, larvae, or both, for each group, and are adapted from Table IV.

A greater proportion of the *Decerebrate* worms contained larvae which had progressed to each of the selected developmental stages, as compared to the other four groups. Thus, of the worms considered, 90% of the *Decerebrate* group contained larvae which had progressed to the trochophore stage, as compared with 60% for the *Dorsal Implant and Dorsal* and *Ventral Implant* groups and 33% for the *Ventral Implant* group. The one worm which had larvae in the *Intact Implant* group contained cleavage stages; the remaining members of this group contained oöcytes. Although the actual numbers are somewhat small for comparison, the data indicate that the larvae of worms in the *Decerebrate* group showed the most developmental progress, the *Intact Implant* worms showed the least progress, and the three groups which received implants of the brain fragments were intermediate in this respect. Of the latter three groups, the *Ventral Implant* showed the least developmental progress, since a smaller proportion of its members had larvae which had progressed to trochophore or later stages.

DISCUSSION

In many nereids, gametic maturity is accompanied by a profound somatic metamorphosis into an epitokous swimming form known as a *Heteronereis* (Clark, 1961). However, even in species that reproduce in the atokous form, several somatic changes are correlated with reproductive maturation. Dales (1950) has described these changes for *N. diversicolor,* an atokous, oviparous species. The coelomocytes fill the body cavity during the early phase of sexual development and almost disappear during the final stages of gametic maturation as the coelom becomes occluded with mature oöcytes. Histolysis and dedifferentiation of the musculature have rendered the body wall thin and fragile at the time of spawning.

N. limnicola, in common with *N. diversicolor,* reproduces in the atokous form. However, the temporal relationship between events characteristic of somatic and gametic maturation differ significantly in the viviparous *N. limnicola* from the general nereid pattern. Histolysis of body musculature does not coincide with the maturation of the gametes. Furthermore, the coelomocytes fill the coelomic cavity of worms containing mature gametes and the coelomocyte concentration does not decrease until after cleavage stages are present in the coelom (Smith, 1950). The body musculature undergoes histolysis during the period when advanced larval stages are present in the coelom. The coelomocyte concentration decreases significantly by the time of parturition. Thus, the muscle histolysis and the reduction of coelomocytes are delayed to a significant extent with respect to the maturation of the gametes, and occur in correlation with the intracoelomic development of the offspring rather than in association with the final stages of gamete maturation, as is the case with oviparous nereid species.

In *N. limnicola* the gametic and somatic aspects of maturation are out of phase, and this shift can be interpreted in the context of present views of the endocrine integration of nereid reproductive development. Both somatic and gametic maturational changes occur simultaneously in other nereid species, and the declining titer of a single inhibitory hormone is thought to control both processes (reviews by Clark, 1965; Durchon, 1967). The lack of synchrony between these processes in *N. limnicola* raises the possibility that the gametic and somatic aspects of maturation may be controlled by separate inhibitory hormones which are withdrawn simultaneously in an oviparous nereid but withdrawn sequentially in *N. limnicola,* a viviparous species. An alternative and more plausible explanation would attribute this lack of synchrony to different sensitivities of the respective processes to the declining titer of a single inhibitory factor. Thus, somatic maturation may have become more sensitive to the low hormone levels at sexual maturity and thus be delayed with respect to gamete maturation, or the gametes may have developed less sensitivity to the inhibitory hormone and mature precociously with respect to the onset of the somatic changes. It is not possible to decide on one of these alternatives on the basis of present knowledge.

The condition of having the somatic and gametic maturational changes occur out of phase is interpreted as a variation of a basic endocrine control mechanism found in oviparous nereids. In the case of *N. limnicola,* this control is adapted as a specialization for a viviparous mode of reproduction. Thus, the endocrine integration of the process of muscle histolysis, which prepares the sexually mature oviparous nereid for spawning, is modified in *N. limnicola* to prepare the worm for parturition.

With respect to hormonal control of gamete maturation, *N. limnicola* does not appear to depart from the general nereid pattern. The two decerebration experiments demonstrate that brain removal results in the premature appearance of larvae in the coelom. In experiment 1, the only two strictly comparable groups are the *Decerebrate* and *Implant,* and these give clear indication of an inhibitory influence on gamete maturation and, therefore, indirectly on larval production. The difference between the *Intact* and *Implant* groups is difficult to interpret, although comparable differences have been reported with respect to the progress of regeneration in *N. diversicolor* with intact and implanted brains (Golding, 1967a). This difference is not confirmed by the larger scale experiment 2, but this latter experiment does confirm the results of experiment 1 which demonstrate that the supraesophageal ganglion exerts an inhibitory endocrine control over maturation of the gametes.

Numerous studies have shown that gamete maturation is inhibited by a hormone presumably secreted by the brain, and this inhibition has been described in males (Durchon and Schaller, 1964; Malecha, 1967) and females (Clark and Ruston, 1963; Durchon and Dhainaut, 1964; Durchon and Boilly, 1964; Hauenschild, 1966; Dhainaut and Porchet, 1967) among several species of oviparous nereids. It is not surprising, therefore, that hormonal inhibition of both sperm and oöcyte maturation occurs simultaneously in *N. limnicola,* a self-fertilizing hermaphrodite in which the eggs and sperm develop simultaneously within the coelom of an individual. The maturation-inhibiting hormone is considered identical to the endocrine factor necessary for regeneration of immature worms (Golding, 1967c). Therefore, the observation that sexually-mature *N. limnicola* does not regenerate is further evidence that, relative to immature worms, the titer of the maturation-inhibiting hormone is low in worms with mature gametes.

There has been some doubt regarding the viability of oöcytes whose growth had been accelerated in response to decerebration, since previous workers have failed to obtain normal development following fertilization of these oöcytes (Choquet, 1962; Clark and Ruston, 1963). However, the present results demonstrate that the precociously-mature oöcytes of *N. limnicola,* produced in response to decerebration, are capable of normal development. It is interesting that normal, mature oöcytes of *N. limnicola* resemble the abnormal oöcytes obtained by Clark and Ruston (1963) following decerebration of *N. diversicolor.* In both cases, the oöcytes lack the dense accumulation of yolk droplets characteristic of most nereid oöcytes, which are normally fertilized externally. However, in *N. limnicola* these oöcytes are fertilized in the coelom, and the coelomic milieu undoubtedly provides the nutrients and other factors necessary for the successful growth and development of the embryos. It has been suggested that the infracerebral gland *a* cells may be involved in this respect (Baskin, 1970).

Smith (1950) reported that the oöcytes of *N. limnicola* (from a different population than those used in this study) normally matured at a diameter of 120–170 μ, and our observations indicated that in the worms from Lake Merced, they were fertilized at about 150–160 μ. In the *Decerebrate* group of experiment 2, the oöcytes of worms whose largest oöcytes were between 73 and 128 μ underwent accelerated growth as evidenced by the precocious appearance of larvae in the coelom. It could not be determined, however, whether the oöcytes had attained a normal size at fertilization, or whether they were fertilized at a smaller diameter. One decerebrate worm, whose largest oöcyte were initially about 86 μ, had 148 μ oöcytes after three weeks. Many of the worms which contained early embryonic stages also had oöcytes of 140–160 μ. These observations suggest that the oöcytes accelerated growth to a relatively normal size before being fertilized, rather than being fertilized at a smaller diameter. It would be of inteerst to know how early the oöcytes of this species can be accelerated by decerebration and still be capable of normal development.

Decerebration of worms with coelomic larvae did not adversely affect the viviparous embryonic development and growth. In fact, the *Decerebrate* group had a greater proportion of worms containing larvae as compared to the *Intact* and *Implant* groups. However, the number of larvae produced by each worm was not recorded, and it is thus conceivable that the groups differed as to the number of larvae produced per worm. The significance of not finding larvae in some of these worms at the conclusion of the experiment is not understood. It is unlikely that the adults underwent parturition, since this event is catastrophic and the adult usually dies. The worms were not fed during the experiment, and it is possible that the larvae were resorbed, since larval resorption has been previously reported in this species (Smith, 1950). Although this question is not resolved, the phenomenon does not alter the general conclusion that larval development *per se* does not require the presence of the cerebral ganglion of the adult.

The results of the experiments in which parts of a brain were implanted into decerebrate worms are significant in view of the problems regarding the presumed source of the cerebral endocrine activity. Previous experimental work has localized this endocrine activity to the posterior region of the cerebral ganglion (Durchon and Dhainaut-Courtois, 1964; Hauenschild, 1966; Golding, 1967b), and attention has been focused upon presumed hormonogenic neurosecretory cells. In contrast, Golding, Baskin and Bern (1968) and Dhainaut-Courtois (1968a) have suggested that the infracerebral gland, which is located on the postero-ventral surface of the ganglion, may fulfill this endocrine role. The present histological observation that the infracerebral gland epithelium was present and normal in appearance on implanted cerebral ganglia that had been secreting the maturation-inhibiting hormone, is compatible with the latter view.

Parts of a brain were implanted into decerebrate hosts to determine whether the source of the maturation-inhibiting hormone is associated with the dorsal region, in which are located many neurosecretory cells, or the ventral region, on which is located the infracerebral gland epithelium. Of the three groups which contained implanted brain fragments, developmental progress was essentially similar in each. As compared to the *Dorsal Implant* and *Dorsal and Ventral Implant* groups, the *Ventral Implant* group showed slightly less developmental progress, which might indicate that the ventral region of the brain exerts a stronger inhibitory effect on maturation. But an equivalent effect was not obtained when a ventral and dorsal part were implanted together; thus, any conclusions about a differential influence of the dorsal and ventral regions, respectively, are considered tentative.

The inhibitory effect of the brain fragments was not equivalent to that of an intact ganglion, even where dorsal and ventral fragments of the same brain were implanted together. But neither were the fragments totally without effect, since all three groups which received an implanted brain fragment showed evidence of developmental retardation when compared to the decerebrate group. This retardation is considered an indirect effect stemming from the inhibition of oöcyte maturation, and does not suggest a direct inhibitory influence on larval development.

Although this experiment did not localize the source of the hormone, the results are consistent with the hypothesis that neurosecretory cells of the brain and the cells of the infracerebral gland epithelium may form a functional unit or system for the production of the maturation-inhibiting principle. The physical integrity of this system was apparently disrupted by transecting the brain so as to separate the infracerebral gland epithelium from its neurosecretory component, resulting in the reduction, but perhaps not complete absence, of hormone secretion.

This interpretation does not conflict with the view that the infracerebral gland cells may be endocrine, although their significance in this respect is incompletely understood. While the *a* cells of the infracerebral gland epithelium have been compared in their ultrastructure to that of endocrine cells of the insect corpus allatum (Dhainaut-Courtois, 1968a), it has been concluded elsewhere that the *a* cells are probably not the source of known endocrine principles (Baskin, 1970). However, the granule-filled *b* cells, which resemble protein-secreting gland cells in their ultrastructure, are most differentiated in immature worms and degenerate in older worms (Dhainaut-Courtois, 1968a). Since this degeneration parallels the disappearance of the maturation-inhibiting hormone, it is tempting to speculate that the *b* cells may be associated with the secretion of this hormone.

The functional significance of the infracerebral gland epithelium and its relationship to neurosecretory activity of the brain remain to be elucidated. Nevertheless, future investigations on nereid endocrinology must consider the possible importance of this system to neuroendocrine regulation in these worms.

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SUMMARY

1. The relationship of gametic and somatic maturation in the atokous polychaete, *Nereis limnicola,* differs significantly from the general nereid pattern. In this viviparous species muscle histolysis and the decrease in the coelomocyte concentration are delayed with respect to the onset of gamete maturation, and occur, instead, in association with the intracoelomic development of the offspring. However, worms with mature gametes are unable to regenerate lost caudal segments.

2. The cerebral ganglion of *N. limnicola* exerts an inhibitory influence on the maturation of sperm and oöcytes. Brain removal results in the precocious appearance of normal larvae in the coelom. This effect is prevented by implanting a brain into the coelom of the decerebrate worms.

3. Histological examination of brains that had been implanted for three weeks revealed that the infracerebral gland epithelium was present and normal in appearance.

4. Decerebration of worms containing coelomic larvae did not delay or accelerate larval development.

5. The effect of implanting parts of brains on gamete maturation of decerebrate worms was studied, and the results indicated that the dorsal part of the brain, which contains many neurosecretory cells, and the ventral part of the brain, on

which is located the infracerebral gland epithelium, had approximately equivalent inhibitory activity when implanted alone or together, but this inhibitory effect on oögenesis was not as great as that produced by an implanted intact brain. These results are interpreted to mean that the infracerebral gland epithelium and neurosecretory cells of the brain may form a neuroendocrine system, the integrity of which is essential for secretion of the maturation-inhibiting hormone at normal levels.

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INTERACTION OF FOOD AND PHOTOPERIOD IN THE TERMINATION OF LARVAL DIAPAUSE IN *CHAOBORUS AMERICANUS* (DIPTERA: CULICIDAE)[1]

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The role of food in the termination of diapause is probably without significance in the many diapausing insects which are inactive and which seldom, if ever, feed. There are, however, certain nematocerous Diptera which overwinter as larvae and are capable of both movement and feeding. Indeed, studies concerning the photoperiodic induction of development in diapausing larvae of the chironomids *Metriocnemus* (Paris and Jenner, 1959) and *Chironomus* (Engelmann and Shappirio, 1965) or of the culicids *Anopheles barberi* (Baker, 1935), *Anopheles bifurcatus* (Vinogradova, 1964), *Anopheles plumbeus* (Vinogradova, 1962), *Aedes triseriatus* (Love and Whelchel, 1955), and *Toxorhinchites rutilus* (McCrary and Jenner, 1965) involved the effects of photoperiod on fed animals only. Studies concerning the overwintering larvae of the culicid *Chaoborus americanus* (Bradshaw, 1969) considered the effects of photoperiod on both fed and starved animals. The assessment of the contribution of food is difficult, if not impossible, in filter feeders or detritus eaters like the chironomids or the culicine and anopheline mosquitoes. Chaoborine and toxorhinchitine mosquitoes, on the other hand, are carnivores. In *Chaoborus,* food and photoperiod have been shown to interact synergistically to effect the termination of larval diapause (Bradshaw, 1969). Furthermore, development in *Chaoborus* is proportional to the number of long days with food. *Chaoborus,* therefore, is conveniently adapted for the study of the food component in the termination of diapause.

MATERIALS AND METHODS

Animals and general conditions

All experiments in this research involve large yellow larvae of *Chaoborus americanus* Johannson caught in George Pond, a small kettle hole in the center of the University of Michigan's George Reserve, near Pinckney, Michigan, during January and February, 1967 and 1968. The larvae were transferred on the day of capture to maintenance conditions of short day (8L, 16D) and 5° C without food. Large numbers of larvae were kept in open gallon jars under these conditions until the starting date of the experiment at which time they were removed, the large yellow larvae, known to be the fastest responding morphs in the population (Bradshaw, unpublished observations), were sorted from this stock, warmed to 25° C, and placed on the experimental conditions outlined below. Food in all cases consisted of laboratory larvae of the mosquito, *Culex pipiens*, provided in saturating amounts unless otherwise stated. All the experiments were run at 25° C under either long day (16L, 8D), or short day (12L, 12D) conditions.

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Statistical methods

Standard methods (Snedecor, 1956, Ch. 6, 7) were used to derive regression lines, regression coefficients (r), and the probabilities of correlation (P). Percentage data were also analyzed by computation of the normal variate as outlined by Ractliffe (1967, pages 136–137).

Comparisons of sample means were made either by the standard t test or by a modification of Duncan's Multiple Range Test (Duncan, 1955). Analysis of variance was carried out with raw data only when appropriate as demonstrated by Bartlett's test for homogeneity of variance (Snedecor, 1956, pages 285–289); otherwise, arcsin-per cent-square-root or log transformations were tried until homogeneity of variance was achieved. If the value obtained for F was significant at the 5% level of confidence, then a variation of Duncan's Multiple Range Test was employed to find significant differences between means (D). In the present study, instead of ranking n means according to their magnitudes, Duncan's Studentized value for comparison at the nth rank is used to derive the significant difference (D). This method is more conservative than either the commonly used Least Significant Difference or Duncan's original method but embodies the convenience of the former.

EXPERIMENTAL RESULTS

Effect of various combinations of food and daylength on the large yellow larvae

Large yellow larvae were exposed to short day without food starting 2–2–68 and 3–31–68, to long day without food starting 2–2–68 to 5–11–68 (5 replicates), to short day with food starting 2–2–68 to 5–11–68 (6 replicates), and to long day with food starting 2–2–68 to 5–11–68 (4 replicates). They were exposed to experimental conditions for at least 30 days unless all larvae had either developed or died prior to that time.

As shown in Table I, short day without food failed to elicit any substantial pupation in the large yellow larvae. Either long day without food or short day

TABLE I

Developmental response of large yellow larvae to various combinations of food and daylength in n replicates of 50 larvae each (mean per cent ± standard deviation)

	Per cent Pupation		n
	After 15 days	After 30 days	
Long day fed	81.0 ± 11.2	89.8 ± 2.7	4
Long day starved	6.0 ± 4.4	6.4 ± 4.1	5
Short day fed	14.7 ± 13.8	16.3 ± 13.4	6
Short day starved	0.0 ± 0.0	0.0 ± 0.0	2

with food elicited some pupation but never more than 20% for the former or 40% for the latter. Long day with food, on the other hand, elicited substantial pupation, even after only 15 days. Thus, while either food or long day photoperiod is necessary for some development, a substantial response is not evoked unless the food and long day stimuli occur simultaneously. It would further appear that 90% of all potential development is realized after only 15 days experimental time. For reasons of convenience, 15 days was therefore chosen as the standard assay period to be used in subsequent experiments.

Effect of daylength on total amount of food consumed

On 4–18–68, two groups of 50 diapausing larvae were placed in $\frac{1}{2}$ oz jars, one larva per jar. One group was placed on long day, the other on short day. Each *Chaoborus* larva was offered twelve *Culex* per day for five days. Thereafter, the food was removed and the *Chaoborus* observed for ten additional days. Development was then scored and the experiment terminated. To increase homogeneity of food particle size, only those mosquito larvae which passed through a 2 × 2 mm mesh but not a 1 × 1 mm mesh were used. Each day, the number of mosquito larvae eaten was tabulated, the remainder removed, and twelve fresh larvae added.

Of the 60 mosquitoes offered, each *Chaoborus* larva ate an average of 30 on long day and 32 on short day. On long day, of 45 surviving individuals, the 44 pupating larvae ate an average of 30 mosquitoes while the only non-developing larva ate 36. On short day, of 49 surviving individuals, both the 19 pupating and the 30-non-developing larvae ate an average of 32 mosquitoes. Daylength, therefore, has little or no effect on the amount of food consumed.

Effect of food or long day on sustaining development

The following experiments are designed to determine whether long day alone or food alone following long day with food is stimulatory, neutral, or inhibitory with respect to development induced by long day with food. A series of 10 oz jars was each provided with 50 diapausing larvae. These groups of larvae were then exposed to 0, 1, 2, 3, or 4 long days with food after which time they were placed on short day without food (experiment started 4–18–68 and replicate started 5–11–68), on long day without food (experiments started 4–18—68 and replicate started 5–11–68), or on short day with food (experiment started 5–11–68) for a total of 15 days experimental time after which development was scored and the experiment terminated.

In all experiments, there was a significant correlation between the number of days exposure to long day with food and the amount of subsequent development (Fig. 1). All exposures to one long day with food elicited more development than the control, *i.e.*, 15 short days without food (Fig. 1A), 15 long days without food (Fig. 1B), or 15 short days with food (Fig. 1C). Furthermore, a significant difference was noted between the responses to any two day increment in exposure to long day with food, *i.e.*, 3 or more days exposure to long day with food elicited significantly more development than 1; 4 days elicited more than 2 or 1, *etc.* (Duncan's Multiple Range Test after arcsin transformation: $F = 47.67$; $P < 0.01$; $D = 11.0$).

After subtracting out the control values, there was no significant difference between the amount of development elicited by short day without food, long day without food, or short day with food following long day with food (analysis of variance: $F = 1.2$; $P > 0.25$). It would thus appear that neither long day nor food has any ability to retard or sustain development once it has been initiated.

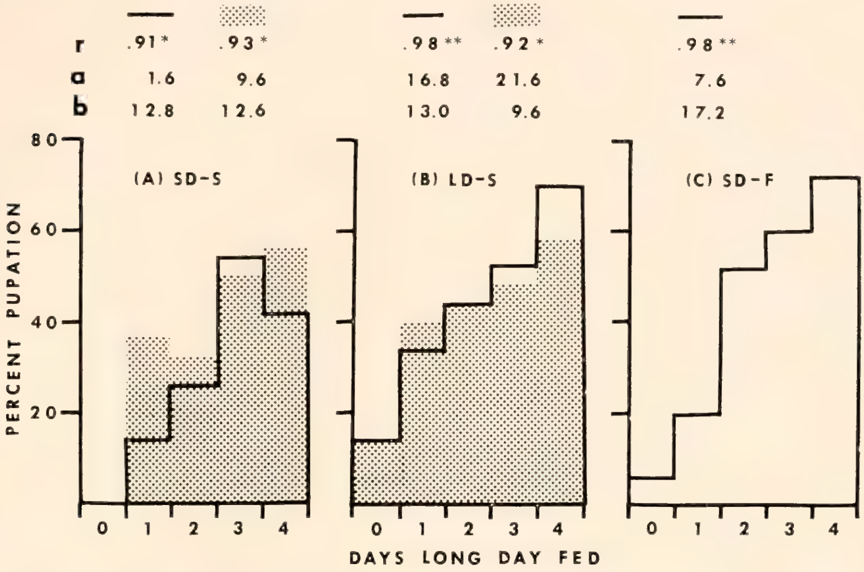

FIGURE 1. Ability of long day and food to sustain development. Diapausing larvae were exposed to long day with food for 0–4 days after which they were placed on short day and starved (A), long day and starved (B), or short day and fed (C), in experiments starting 4–18–68 (solid line) or 5–11–68 (shaded outline). Abbreviations are: r, coefficient of correlation between days long day fed and and per cent pupation; *, significant correlation; ** highly significant correlation; a and b, constants in the regression equation: % pupation $= a + b$ (days long day fed); SD, short day; LD, long day; F, fed; S, starved.

Persistent effects of long day

This experiment was designed to test whether previous exposure to long day without food would enhance the developmental response of larvae subsequently exposed to short day with food. Diapausing larvae were placed in a series of 10 oz jars, 50 animals to a jar. They were then exposed to long day without food for 0 (control), 1, 2, 3, 4, 5, or 6 days after which they were transferred to short day and provided an excess of food for a total experimental time of 15 days after which development was scored and the experiment was terminated. The initial experiment starting 5–11–68 was repeated with fresh animals starting 6–1–68.

Unlike the response to long day with food (Fig. 1), there was no correlation between the duration of exposure to long day without food and the amount of development elicited (Fig. 2, solid line) (after subtracting out the control values: $r = 0.14$; $P > 0.85$ for 10 df). While there were no significant differences among responses elicited by 1, 2, 3, 4, 5, and 6 days exposure to long day without food, 2 and 6 days exposure elicited a significantly greater amount of pupation than did the control (Duncan's Multiple Range Test after arcsin transformation: $F = 5.00$; $P < 0.05$; $D = 12.8\%$). These results suggest the photoperiodic information may be accumulated to some extent by *C. americanus* but that the synergistic interaction of food and photoperiod requires both parameters simultaneously rather than sequentially.

Persistent effects of food

The following experiments reciprocate the ones above and are designed (1) to test whether trophic information is stored by *C. americanus* and (2) to substantiate the inference that the synergistic interaction of food and long day requires simultaneous input. For this purpose, diapausing larvae were placed in a series of 10 oz jars, 50 animals to a jar. They were then exposed to short day with an excess of food for 0 (control), 1, 2, 3, 4, 5, or 6 days after which time the food was removed and the *Chaoborus* larvae transferred to long day conditions without food for a total experimental time of 15 days. The initial experiment starting 4–18–68 was repeated in duplicate, both of the latter experiments starting 5–11–68.

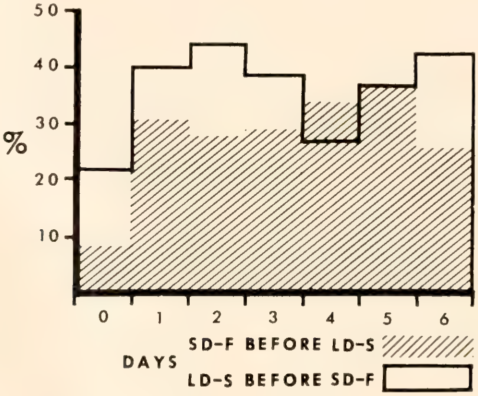

FIGURE 2. Developmental response of larvae on short day with food after 0–6 days exposure to long day without food (average of two replicates) and of larvae on long day without food after 0–6 days exposure to short day with food (average of three replicates); ordinate, per cent pupation; other abbreviations as in Figure 1.

As in the case of long day followed by food, there was no correlation between the duration of exposure to food and the amount of development elicited (Fig 2, diagonal shading) (after subtracting out the control values: $r = 0.07$; $P > 0.99$). On the other hand, all of the experimental values were significantly greater than the controls, even though they were not significantly different from each other (Duncan's Multiple Range Test after arcsin transformation: $F = 3.03$; $P < 0.05$; $D = 13.2\%$). These results confirm the concept that the synergistic response to food and long day requires simultaneous rather than sequential input of these factors.

Inspection of the larval mortality (Fig. 3A, solid dots) and pupal survivorship (Fig. 3B) after 15 days experimental time reveals that both are highly significantly correlated with the number of days feeding on short day. This observation indicates that food is stored—as long as 15 days—and may be called upon to support larval life or adult development.

The following experiments, designed to further elucidate the long-term effects of food, employed the duplicate experiments started on 5–11–68 as described above. At the end of 16 days total experimental time, the duplicates were split into two groups of 7 experimental populations each, having received from 0 to 6 days prior feeding. The sample size in each experimental population then consisted of that number of larvae alive and undeveloped at the end of 16 days. One group was given a one day food pulse, the other a four day food pulse, both under long day conditions. After pulse, the larvae remained on long day without food until a total of 31 days experimental time had elapsed. Larval mortality, pupal survivorship, and amount of development were then scored and the experiment terminated.

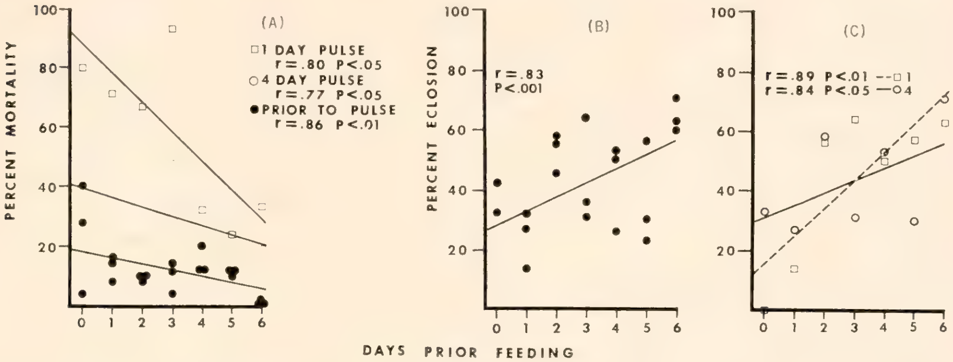

FIGURE 3. Larval mortality (A) and pupal survivorship (B–C) after 0–6 days exposure to food on short day followed by continuous long day without food. Percentages after 15 days experimental time are shown by solid dots ●. On day 16, duplicate experimental groups were provided a one day (□) or a four day (○) food pulse on long day. The food was removed after this time and the larvae again placed on long day without food. Per cent larval mortality and per cent eclosion were scored on the 31st day, using the number of living, undeveloped larvae on day 16 as the sample size.

Among both the one day and four day food pulsed larvae, there was a significant correlation between prior feeding ten to thirty days beforehand and larval mortality (Fig. 3A) or pupal survivorship (Fig. 3C). Development in response to a one day or a four day food pulse also appeared to be proportional to the number of days prior feeding when per cent response was calculated on the basis of the number of larvae alive at the initiation of the food pulse experiments (*i.e.*, the 16th day). But, if percentage development is calculated on the total number of this sample size surviving until the 31st day ($n =$ number of living larvae + number of pupae), then development was no longer proportional to the number of days prior feeding ($r = 0.47$; $P > 0.05$). It would thus appear that food stored from remote feeding is capable of maintaining larval life or sustaining adult development but does not contribute to the termination of diapause in *C. americanus.*

Discussion

Food very clearly is accumulated and stored by the larvae; the effects of this stored food are readily seen contributing to larval survivorship (Fig. 3A) and to adult development (Fig. 3B, C). Yet, these same nutritional reserves do not enable the larvae to respond developmentally to long day in the absence of continued feeding. The apparent inconsistency is easily explained by ascribing a dual role to food in the developmental process. First, food is functioning as an energy source; second, food, or feeding, is acting as a physiological trigger to initiate development. Without long day photoperiod, food still increases the nutritional plane of the larvae but is unable to trigger substantial development. Thus, larvae exposed to food on short day and then exposed to long day without food do not show an increasing response related directly to the number of days prior feeding, nor do they show a food independent synergistic response to long day without food after a certain "critical number" of days prior feeding. Moreover, when careful comparison of food intake is made, it becomes clear that the above failure to respond to feeding on short day is not due to a short day repression or long day stimulation of feeding.

The simplest nutrition-independent explanation of food as a physiological trigger would be stimulation of a neuroendocrine reflex by food as an environmental cue. This model is not novel in endocrine control of development but is paralleled by the control of moulting in the bugs *Rhodnius* (Wigglesworth, 1933) and *Cimex* (Kemper, 1931). In *Rhodnius*, the moulting stimulus is initiated by stretch receptors on the intersegmental muscles of the abdomen which is sizeably distended after a blood meal (Wigglesworth, 1934). Undoubtedly, *Rhodnius* derives nutrition from these blood meals since (1) the moult occurs within a constant time interval after a normal blood meal, regardless of the duration of the fasting period prior to the meal and (2) the animal may continually consume small amounts of blood and yet refrain from moulting indefinitely. The important point is that the induction of moulting by food is effected by independently of these nutritional benefits.

If food in *Chaoborus* is also acting via a neuroendocrine reflex independently of nutritional benefits, then not only should the persistent effects of food stimulation be of short duration as seen above (Fig. 2), but should also be of the same order of magnitude as the persistent effects of long day stimulation. The response of animals exposed to 1 to 6 long days without food before exposure to short day with food bears out this argument (Fig. 2, solid line).

A paradox remains both in the experiment exposing animals to short day with food before long day without food, and in the experiment exposing animals to long day without food before short day with food: some or all experimental animals respond significantly more than do the controls, even though both food and long day only interact synergistically when they occur simultaneously This problem may be clarified if one ascribes a physiological latency to both food and long day. Such a latency is not especially new since diapause in many insects may be cued by stimuli perceived in previous instars or even the previous generation. Thus, in the present case, while the experimental design implies that diapausing larvae are receiving 1–6 days feeding prior to long day, a lag in the larvae themselves gives the physiological impression that food is being perceived from 2–7 days. In all the experimentals, but not in the controls, there will then be one day's overlap during which the larvae receive both the latent food stimulus and the newly impressed long day stimulus; hence, one day's worth of synergistic response.

Essentially, this model defines the period of physiological latency as that time during which synergistic interaction of food with long day is taking place. Thus, the actual duration of food latency is the number of days on long day with food necessary to induce development equal to the average development resulting from 1–6 days feeding on short day before exposure to long day without food. The latter value is 29% development for the experiment starting 4–18–68 and 32% development for the experiment starting 5–11–68. The regression equations for development as a function of days long day fed before long day starved are (for experiments started 4–18–68 and 5–11–68, Fig. 1B):

$$\begin{aligned} &4\text{–}18\text{: } \% \text{ development} = 17 + 13.0 \text{ (days long day fed)} \\ &5\text{–}11\text{: } \% \text{ development} = 22 + 9.6 \text{ (days long day fed)} \end{aligned}$$

Solving the equations for the parameter (days long day fed), given 29% and 32% development, the latent period of food is 0.9 days for both 4–18 and 5–11. Similarly, the latent period of long day is found from the average pupation induced by 1–6 days long day without food before exposure to short day with food in experiments started 5–11–68 and 6–1–68, 42% and 34% development, respectively. The regression equation for the only estimate of inductive capacity of long day with food before exposure to short day with food on 4–18 (Fig. 1C) is:

$$\% \text{ development} = 8 + 17.2 \text{ (days long day fed)}$$

yielding latent periods of 1.5 and 2.0 days. These latter values should be regarded only as estimates since the pertinent experiments were not started concurrently and since this source of error was compounded because the experiments were in progress during that time of year when the effects of long day were declining and those of food increasing (Bradshaw, 1969). Nonetheless, it can be concluded that the latent period of food probably does not exceed that of long day. The latent periods of these two inductive cues thus appear to be similar and completely consistent with the concept of nutritional independence of food in the inductive process. It should be noted that nothing here is meant to imply that food does not make a substantial, long-term contribution to the developing pupa or adult (Fig. 3); the important point to be made again is that this nutritional use of food is independent of the use of food as an environmental signal.

Appreciation is extended to Billy E. Frye, Christina M. Holzapfel, and David G. Shappirio for their consideration and advice concerning the research here presented.

SUMMARY

1. Food and long day photoperiod interact synergistically to effect the termination of larval diapause but the input of food and long day must be simultaneous rather than sequential for the synergistic effect to take place.

2. Daylength does not affect food consumption.

3. Neither food alone nor long day alone is capable of augmenting development initiated by long day with food; likewise, short day without food does not appear to retard development once it has been initiated.

4. Food is nutritionally capable of affecting larval and pupal survivorship for up to several weeks but its capacity to interact synergistically with long day to induce development persists for only a day.

5. The capacity of long day to interact synergistically with food to induce development persists for $1\frac{1}{2}$ to 2 days.

6. Food, therefore, is probably acting via some neuroendocrine reflex as an environmental cue independently of its nutritional contribution to the overwintering larva or to the resultant pupa and adult.

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DIXENIC CULTURE OF *DAPHNIA MAGNA,* STRAUS

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Banta (1921) demonstrated that *Daphnia* could be grown agnotobiotically with relative ease. Since then, rearing was improved by replacing horse-manure infusion with unialgal cultures (Léfèvre, 1942). Large populations of *D. magna* were maintained for 18 months in artesian well water, and fed with yeast and *Scenedesmus;* these cultures were stable and supplied abundant progeny of known age, genetic homogeneity and vigor, for insecticide bio-assays (Dewey and Parker, 1964). Attempts to replace pond water with synthetic salt solutions have met with limited success (Anderson, 1945; Freeman, 1953; Boyd, 1957; Taub and Dollar, 1964). Dewey and Parker (1964) reported that under the most favorable conditions (Wesson's (1932) salt mixture in distilled water) fecundity gradually declined and reproduction ceased at the 32nd generation.

Taub and Dollar (1968) re-explored the dependence of alga-fed *Daphnia pulex* on water biologically conditioned in aquaria (BCW). They concluded that algae grown in defined media were deficient in nutritional factors needed by *Daphnia.* Presumably "BCW" contained unidentified substances, which fostered normal development and ovulation of *Daphnia.*

Since this problem seemed nutritional, we axenized *D. magna* and fed it on pure cultures of algae to define conditions for sustained culture.

MATERIAL AND METHODS

Crude cultures

Agnotobiotic *D. magna* strain #10 was kindly supplied by Dr. F. Taub, College of Fisheries, Seattle, Washington. Stock cultures were maintained in covered "storage dishes" (Corning #3250), 30 individuals in 200 ml of DM_2, a synthetic medium containing (w/100 ml dist. H_2O): KCl, 5 mg; $MgSO_4 \cdot 7H_2O$, 4 mg; Ca (as Cl^-), 2 mg; K_2HPO_4, 0.6 mg; KH_2PO_4, 0.6 mg; $NaNO_3$, 5 mg; $NaSiO_3 \cdot 9H_2O$, 2 mg; Fe (as Cl^-), 0.05 μg; Metals PII, [1 ml of P II metals contains: ethylenediaminetetraacetic acid (as Na_2), 1 mg; Fe (as Cl), 0.01 mg; B (as H_3BO_3), 0.2 mg; Mn (as Cl), 0.04 mg; Zn (as Cl), 5 μg; Co (as Cl), 1 μg; (Provasoli, McLaughlin and Droop, 1957)] 1 ml; Metals S II, [1 ml of S II metals contains: Br (as Na), 1 mg; Sr (as Cl), 0.2 mg; Rb (as Cl), 0.02 mg; Li (as Cl), 0.02 mg; Mo (as Na salt), 0.05 mg; I (as K), 1 μg; V (as NH_4VO_3), 1 μg; (Provasoli, McLaughlin and Droop, 1957)] 1 ml; vitamin B_{12}, 1 μg; and thiamine HCl, 10 μg; adjusted to pH 7.0.

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They were fed axenically grown *Scenedesmus obliquus* (Indiana Collection No. 393) and *Chlamydomonas reinhardi*, minus strain (Indiana Collection No. 90). Continuous cultivation was kept by subculturing the first 30 newborn nauplii of each generation.

Transfer dishes were prepared 8 days previously by inoculating DM_2 with algae grown on DA medium. [DA medium: mg/100 ml: $Na_3 \cdot citrate \cdot 2H_2O$, 2.0; $MgSO_4 \cdot 7H_2O$, 2.0; K_2HPO_4, 2.0; Fe (as SO_4), 0.2; Thiotone (Baltimore Biol. Lab), 60.0; Trypticase (Baltimore Biol. Lab), 16.0; yeast extract (Difco), 5.0; pH 6.5 (DA = Medium A, Table 2, p. 840, Provasoli and Pintner, 1953).] Addition of DA to DM_2 promoted initially rapid growth of bacteria which soon subsided and was succeeded by algal growth. Mortality was high if newborn *Daphnia* were inoculated during the period of bacterial growth.

Antibiotics

Serial washings of nauplii from crude cultures failed to eliminate bacteria. Antibiotics were tried after determining their effectiveness on the bacterial flora association with *Daphnia*. Samples of water from crude cultures were diluted with an equal volume of Dextrose Broth (Difco); 4 hours later 1-ml aliquots were spread on solidified nutrient agar (Difco). One hour later, surplus fluid was removed and antibiotic sensitivity discs (Difco) were pressed on the agar surface. Zones of inhibition, at room temperature, were recorded at 24, 48 and 72 hours. No one antibiotic completely inhibited bacterial growth. Streptomycin sulfate and chloromphenicol were the most effective; however, resistant strains developed within the zones of inhibition in all cases.

Antibiotics effective against the micro-organisms were tested for toxiciity to *D. magna*. Nauplii or adults of *Daphnia* were exposed to varying concentrations of antibiotics in 10 ml of DM (= DM_2 without thiamine and B_{12}). Organic substances were excluded to avoid excessive growth of resistant bacteria. The antibiotics were dispensed from fresh Seitz-filtered stock solutions. *Chlamydomonas reinhardi* and *Scenedesmus obliquus* were added as food. The cultures were observed twice daily. Inhibitory concentrations of antibiotics paralyzed the second antennae of the *Daphnia*, the animals settled to the bottom of the tube and soon died.

Single antibiotics permitting survival of adults and larvae for > 2 days were (μg/ml): chloramphenicol 25; kanamycin 100; nalidixin 50; neomycin 20; polymixin 7.5 (1000 μg = 7760 units) penicillin 500 (1000 μg = 1650 units); streptomycin-SO_4 100; tetracycline 10; trichomycin 50.

Several mixtures designed to suppress a wide bacterial spectrum were prepared and tested. Chloramphenicol, kanamycin, nalidixin and polymixin were omitted because few *Daphnia* exposed to them survived. The concentration of penicillin was kept below 450 μg/ml because larger doses changed the pH of the media to < 5.6—a pH not tolerated by *Daphnia* in these media.

Axenization of Daphnia magna

In crude cultures *D. magna* nauplii became females after 8 days and deposited their first parthenogenetic brood (4–6 larvae per female). Seven-day-old gravid females with eggs ready to hatch were transferred to tubes containing 10 ml of sterile DM and 0.5 ml of antibiotic mix D [antibiotic mix D contained (μg/ml): neomycin 10; streptomycin 1500; penicillin 6000 (1000 μg = 1650 units); tetracline 400]. This concentration, although lethal to adults, allowed 2–3-day survival —an ample time for release of neonates.

To prevent fecal accumulation and overgrowth by bacteria, the females were transferred every 15 minutes to fresh tubes similarly prepared. Usually the nauplii hatched during the same working day; if not, gravid females were left overnight in DM and 0.25 ml/10 ml of antibiotic mix D. Alternatively, sublethal doses of antibiotic mixtures (0.1 ml/10 ml), were used; obviously more rinses were needed to remove by dilution the bacterial flora.

Neonates collected within minutes after deposition were washed through 10 serial baths (10 ml DM + 0.25 ml antibiotic mix D). The animals remained in each bath 10 minutes. After the 10th wash, sterility was tested by transferring them into DA liquid medium for ½ hour [This tube was incubated in the dark at 28° C. If infected, visible turbidity appeared before algal growth could mask it. Darkfield microscopy was also used to detect infectants.]; then they were distributed singly into tubes with 10 ml of DM_2. Several food algae were added aseptically alone and in combinations to find an adequate food for axenic cultures.

Maintenance of axenic stock cultures

Bacteria-free nauplii developed into fertile females readily when fed *S. obliquus* and *C. reinhardi* grown in DA medium. To maintain dixenic cultures, filial generation nauplii were collected and washed three times in 10 ml of DA liquid medium; the last wash tube served as sterility test. The washed larvae were distributed singly in screwcap tubes (20 × 125 mm Pyrex) containing 10 ml of DM_2 and inoculated with 0.5 ml each of dense cultures of the two algae grown in liquid DA. The tubes were incubated at room temperature in racks illuminated (200 ft-c) continuously by white fluorescent lamps. This initial inoculum of algae sufficed to feed the larvae and to produce ample algae for feeding *ad libitum* the adult female for 20 days if each newborn brood was withdrawn within a day of deposition. Every 30 or so generations, records of survival, developmental time, and fecundity were made on a set of seven tubes. Survival of nauplii was 90–100%; of these, 90–95% became fecund females in 8 days. The average production was 29.7 nauplii per female life-span. After the 20th day, the algal population became too small to support *Daphnia* fertility. Over 200 generations of *Daphnia* have been obtained to date without lessening of vitality.

Several variables that may have influenced cultures were studied. In each case nauplii were transferred consecutively into six 10-ml tubes of DM to minimize carry-over of nutrients and to eliminate the algae, before being inoculated into the experimental tubes.

RESULTS

Experience with mineral media for lake algae indicates that they require dilute media (Chu, 1942; Rodhe, 1948; Provasoli and Pintner, 1953). Although optimal growth depends, roughly, on Ca/Mg, Na/K and divalent/monovalent ratios being optimal, planktonic algae adjust well to wide variations provided that tolerance limits in respect to total solid concentrations are not approached (Provasoli, McLaughlin and Pintner, 1954). Chu (1942) simply diluted the old medium of Benecke, then determined the best ratios for planktonic algae. Murachi and Imai (1954) found that a slightly modified Bristol solution diluted 10 × was satisfactory for *Moina macrocopa.*

Several dilute media for freshwater algae were tried. Various modifications and combinations of the more promising media led to medium DM ($= DM_2$ minus vitamins), which supported good algal growth without injuring *Daphnia.* These trials were done with bacterized cultures of *Daphnia.* Under such conditions *D. magna* fed *Scenedesmus* survived only a few generations. Other food organisms were tried. The combination *S. obliquus* and *Chlamydomonas reinhardi* seemed best: it allowed 30 non-axenic generations before fertility decreased. [These two algae were grown in DM medium and inoculated in new DM a few days before transferring the newborn *Daphnia* of the next generation.]

In several cases, for lack of well-grown algal cultures, we used, as inoculum algal cultures from the culture collection which were grown on agar slants of DA medium; the organic components do not harm algal growth and permit detection of contamination. Because of the organic content of the medium, care was taken at first to remove the algae without digging into the agar since carry-over of nutrients might lead to excessive bacterial growth. On the contrary, introduction of DA agar flakes resulted in better growth of *Daphnia.* This observation became useful when fertility of *Daphnia* declined; large flakes of DA agar were added with the algal inoculum and restored fertility. Later on 0.5 ml of each algal culture grown in DA broth was used as inoculum. Thirty additional non-axenic generations were obtained before maintenance of the bacterized strain was discontinued.

Meanwhile axenic newborn specimens of *D. magna* were obtained. Several potential food organisms were tried which had been grown on DA agar. Inoculated axenically into DM_2 medium, *Chlorella vulgaris* and *C. elipsoidea* supported development to young females but not reproduction; *Navicula pelliculosa* and *Saccharomyces cerivisiae* allowed maturation of adults and egg production, but the eggs never hatched. *Scenedesmus quadricaudatum* and *Chlamydomonas moewusii* permitted only survival of larvae (1–3) days). *S. obliquus* and *C. reinhardi,* used singly, supported growth up to young females; increasing the light from 100 to 200 ft-c resulted in adult females but no reproduction. *S. obliquus* at 200 ft-c produced substances which thickened the medium; as a result, the specimens of *Daphnia* were immobilized and soon died. *D. magna* fed on the combination *S. obliquus* and *C. reinhardi* in DM_2 gave adult females at 100 ft-c and finally nauplii at 150 and 200 ft-c. Surprisingly the presence of *C. reinhardi* resulted in no gelling of the medium by *S. obliquus.*

Ten generations of *D. magna* were obtained in DM_2 with the 2 algae as food, then fertility fell off sharply. By that time we had found with bacterized cultures that the addition of DA (1 ml/10 ml medium) restored fertility. This held even under germ-free conditions. We are now a the 200th germ-free generation; mean generation time 8.5 days; mean survival of fecund females 20–22 days; mean newborn production per female life-span 30 (Table I).

Agitation and higher concentrations of nitrates and phosphates were tried to eliminate the possibility that the organic enrichment simply made good a deficiency of the medium in these nutrients. In media without vitamins (DM), increase in total phosphates up to 4 mg% and nitrates to 40 mg% resulted in ovigerous females (as opposed to young females with lower concentrations of N and P) but the eggs generally did not hatch and the few nauplii obtained were sickly. Addition of vitamin B_{12} and thiamine (DM_2), DA or liver extract resulted in viable nauplii. Several modifications of DM_2 were then tried (Table I). Higher N and P and/or the addition of an N-containing pH buffer failed to support more generations even in the presence of B_{12} and thiamine.

TABLE I

Modification of Basal media

(*food algae* S. obliquus *and* C. reinhardi)*

	Media designation (mg%; w/v)						
	DM_2	DM_3	DM_2 +DA	DM_5	DM_5 +G	DM_5	DM_8
P (as K_2HPO_4 and KH_2PO_4)	0.24	0.24	0.24	0.24	0.24	0.81	0.81
N (as $NaNO_3$)	0.82	0.82	0.82	0.82	0.82	3.3	3.3
N (in 30 mg% glycylglycine buffer)		6.3					
Thiamine (µg%, w/v)	10	10	10		6.3		10
B_{12} (µg%, w/v)	0.1	0.1	0.1				0.1
Yeast extract			0.5				
Thiotone (Baltimore Biol. Lab.)			6.0				
Trypticase (B.B.L.)			1.6				
Daphnia magna							
Generations**	12	12	>200	1	1	1	10
Generation time***	9.8	8.6	8.5 [6 11]	9	9	9	12.9
Mean new born/♀****	15.6	23	29.7 [10 66]	9	5	2	21.3

* Algae grown separately in various liquid media and fed to *Daphnia* being cultured serially in corresponding media.

** Consecutive generations obtained.

*** Days needed by a newborn nauplius to become a fertile female.

**** Average of several trials.

Since the only factors inducing sustained, if limited fertility were vitamin B_{12} and thiamine, other vitamins were tried as replacements of the organic enrichment of the DA medium. Since the growth and fertility of *Daphnia* apparently depended upon the composition of the medium in which the algae were grown, depleted algae grown serially in a medium without vitamins (medium DM) when inoculated with *Daphnia* nauplii in media with vitamins might show a substantial difference in number of F_1 nauplii. This was indeed so: the effect of various vitamins was evident in 3 weeks. The only vitamin which significantly increased the F_1 nauplii was B_{12}. Combined with B_{12} and thiamine, pantothenate increased nauplii production (Experiment A, Table II); pyridoxine was occasionally effective.

To confirm these short-term results, in Experiment B (Table II), the algae were again grown without vitamins (DM_7) and subjected to the experimental variables, reduced after the F_1 to one optimal concentration for each vitamin. The

TABLE II

*Effect of vitamins (No. nauplii/♀)**

Vitamin addition ($\mu g\%$)	Exp. A F_1 only	Exp. B												Exp. C†
		F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{15}	F_{20}	
Mineral base (DM_7)	2	0.7	0											0
$DM_7 + B_{12}$ 0.05		8.3	9	discontinued										
$DM_7 + B_{12}$ 0.1	18	21	26	24	26	18	8	1.7	0.3	0				26
$DM_7 + B_{12}$ 0.2		19	22	discontinued										
DM_7 + thiamine 25		0												
DM_7 + thiamine 50	2.5	4.5	0											0
DM_7 + thiamine 100		2.3	0											
DM_7 + pantothenate 12**		0												
DM_7 + pantothenate 25**	0	0												0
DM_7 + pantothenate 50**		1.3	0											
$DM_7 + B_{12}$ 0.1 + thiamine 50	20	23	24	22	15	11.3	7	0						23
$DM_7 + B_{12}$ 0.1 + pantothenate 25	32	25	22	17.3	3	0								31
$DM_7 + B_{12}$ + thiamine + pantothenate 25***	44	33	29	35	32	28	36	31	31	28	31	26	28	45
$DM_7 + B_{12}$ + thiamine + pantothenate + pyridoxine****								28	32	30	33	26	31	
$DM_7 + B_{12}$ 0.1 + thiamine 50 + DA (1 ml/10)		29	38	41	28	37	38	36	37	31	31	31	33	

* Each value in table is the average of triplicate tubes.

** Ca·pantothenate was filter-sterilized and added aseptically.

*** The No. nauplii given for F_1–F_5 are for the aseptic addition of 25 $\mu g\%$ of Ca·pantothenate. From F_6 on, the values are for 250 $\mu g\%$ Ca·pantothenate autoclaved with the medium. The generations F_6–F_{10} were run in duplicate series (*i.e.*, these 2 concentrations of pantothenate) of triplicate tubes; the average number of nauplii per female was almost identical indicating that autoclaving resulted in a high inactivation of pantothenate. The series with aseptic addition of pantothenate was discontinued at the F_{11} which became infected.

**** This series was inoculated with nauplii, of the F_6 in B_{12} + thiamine + Ca·pantothenate.

† See text.

previous results were confirmed: only B_{12} + thiamine + pantothenate supported fertility beyond the F_8, and the average number of nauplii per female life-span was similar but consistently lower than with $DM_7 + B_{12}$ + thiamine + DA (1 ml/10 ml)—a combination similar to DM_2 + DA which supported over 200 aseptic generations. Most of the effect of the organic enrichment could thus be replaced.

The fact that in these experiments the algae and *Daphnia* were grown in the same medium made it difficult to discern whether the organic enrichment (or the vitamins) acted directly on the crustacea or *via* the algae. To rule out the possibility that *Daphnia* fertility depended upon direct uptake of organics from the medium, the algae were grown separately on agar media to avoid carry-over of enrichment, then fed to *Daphnia* in a completely mineral medium (DM). The original experiment (Table I) of comparing DM_2 and DM_2 + DA was repeated: *C. reinhardi* and *S. obliquus* were grown seperately on DM_2 and in DA solidified with 2% agar. When grown, a loopful of each alga was scraped from the agar surface, avoiding removal of agar pieces, and inoculated with one *Daphnia* nauplius in a tube of DM (Experiment C, Table II). Additional loopfuls of algae were added if needed. The line fed on algae grown in DM_2 failed to reproduce beyond the 11th generation. The line fed on algae grown in DA showed undiminished fertility (the experiment was discontinued at the 20th generation), duplicating the results obtained when liquid DA was added directly to the algae-crustacean culture.

TABLE III

Effects of algal preconditioning

Experiment	Medium used for culturing				Average results*		
	algal inoculum		algae + *Daphnia*		Nauplii ♀	Generation time (days)	No. generations averaged
	Mineral part	Enrichment	Mineral	Enrichment			
B	DM_7 liquid (no vitamins)	0	DM_7 +	B_{12} + thia. + pantoth.	31	12.6	15
			DM_7 +	B_{12} + thia. + DA medium	34	13.7	15
			DM_7	0	0.7	17	1
C	DM agar[2]	B_{12} + thia.	DM	0	18.4	8.5	11
	0	DA agar†	DM	0	19.5	8.6	20
Culture maintenance	0	DA liquid	DM	B_{12} + thia. + I ml/10 DA	29.7	8.5	50

* Average of data from one or more generations; for each generation the results of triplicate tubes were also averaged.

† Algal cells only were removed from agar and served as inoculum.

DISCUSSION

Numerous attempts to replace pond water with defined salt solutions for continuous cultures of daphnids have failed (at best only 30 consecutive generations of *D. magna* were obtained by Dewey and Parker). We experienced a similar failure in DM medium. Later results show that for continuous cultures of *D. magna*, choice of food organisms and, especially, provision of an organic enrichment are far more important than the composition of the mineral medium; our medium DM does not differ very much from medium 37 of Taub and Dollars (1968).

Fourteen species of Cladocera are now in monoxenic continuous culture with *C. reinhardi* as food organism in a unique medium consisting of Ca acetate, albumin, water-soluble vitamins, trace metals, and distilled water (Murphy, 1970); no advantage was found in adding other mineral salts! An explanation of this astonishing feat is simply that *C. reinhardi* is a collector of essential minerals (see composition in table 5, page 615, Taub and Dollar, 1968).

It is not surprising, therefore, that other daphnids thrive on DM_2 enriched with pantothenate; many bacterized generations of *D. galeata mendotae* (J. S. Suffern, Biology Dept., Yale University, personal communication) and >10 bacteria-free generations of *D. pulex* and *S. mucronata* (D. E. Conklin, Haskins Labs., personal communication) were obtained without loss of fertility when fed in this medium on *C. reinhardi* and *S. obliquus.*

Addition of vitamins to inadequate algal food had also been beneficial for *Tigriopus japonicus* (Shiraishi and Provasoli, 1959) but these findings were based on only a few generations. The results of Murphy attest that addition of vitamins is essential in supplementing the nutritive value of one alga for several daphnids. As in our experiments, vitamin B_{12} and Ca pantothenate had a decisive effect on female fertility and viability of nauplii, and permitted, with the addition of thiamine (= his basal medium), over 40 generations of *Daphnia pulex, Scapholeberis mucro-*

nata and *Simocephalus serrulatus* fed on *C. reinhardi* and *S. obliquus*. However this medium failed to support continuous culture of other daphnids until a mixture of 8 additional vitamins was added. This addition improved also the number of nauplii produced in the 1st brood of the above 3 species. In other experiments *C. reinhardi* could be employed as the sole food organism for 14 species by increasing the concentration of choline, pyridoxal, inositol, riboflavin, and nicotinamide. In this medium, *D. magna, S. serrulatus* and *D. retrocurva* were the most difficult to maintain, indicating that some daphnids may be nutritionally more exacting than others.

Previous experiments on *Artemia* (D'Agostino and Provasoli, 1968; Provasoli and D'Agostino, 1969) gave circumstantial evidence that organic enrichment influenced the fertility of *Artemia* (*i.e.*, number of generations) not directly but *via* the algae. The results with *D. magna* grown in mineral media and fed with algae which had been grown separately on organic enrichments or vitamins (Experiment C) seem to finally prove this hypothesis.

Experiments B and C support another hypothesis, *i.e.*, that the enrichments modify the nutritive value of the algae for *Daphnia*. Generation time (*i.e.*, days needed for a nauplius to become a fertile female) depended upon the kind of medium in which the algae used as inoculum were grown (Table III).

If the 2 algae were pregrown for several transfers in mineral media and inoculated in a rich medium with one *Daphnia* nauplius (Experiment B), the *Daphnia* generation time was long (12–13 days) but the number of nauplii per female was high (30–34). Conversely, if the algae were pregrown in organic media (DA or DM_2) and inoculated in mineral media (Experiment C) the generation time was short (8.5 d) and nauplii production per female was low (18–20).

The preconditioned algal inoculum, once transferred in a new medium with one *Daphnia* nauplius, starts to reproduce logarithmically; the medium greens. Simultaneously, the nauplius as it grows to a fertile female, grazes more algae. The algae, as they divide in the new medium, change in physiology and storage products, either losing gradually their nutritional value (if preconditioned in organics and inoculated in mineral media) or gaining nutritional value in the converse experiment. As it happened, the generation time should therefore be influenced by the type of medium in which the algae were pregrown, and production of nauplii by the type of medium in which the algae have been inoculated with the *Daphnia* nauplius. Since the difference in generation time between experiments B and C was of 4.5 days, the size of algal inoculum and/or rate of division of the algae were probably high. A closer equilibrium between grazing rate and algal division rate—grazing rate tending to nullify the positive or negative nutrient effect of algal division—should result in a much smaller differential in generation time. Naturally, the differential may also indicate that the medium-mediated physiological changes in the algae leading to a different cell composition are slow. The short generation time and high nauplii production obtained for 200 generations when the algae were pregrown (DA) and inoculated (DM_2 + DA) in organic enriched media confirms experiments B and C.

That the enrichment acts *via* the algae and not directly on the crustacea is further substantiated by the recent demonstration that crustacea are extremely inefficient in the uptake of solutes (Anderson and Stephens, 1969) and that because of this, *Artemia salina*, when grown *in vitro* on artificial food, is unable to grow unless the major nutrients are presented as particles (Provasoli and D'Agostino, 1969).

Since the results of Murphy (1970), Lewis (1967) and of our group indicate that widely differing genera of freshwater, euryhaline and hyperhaline filter-feeding crustacea behave similarly, it seems probable that addition of vitamins to 2- or 3-membered algae-crustacea cultures might permit continuous cultivation of crustacea which so far have proved difficult to grow.

SUMMARY

1. *Daphnia magna* can be grown for at least 200 generations, axenically or in crude cultures, in a defined mineral medium, enriched with vitamin B_{12} and thiamine and 1 ml/100 of a dilute organic medium, when fed with *Chlamydomonas reinhardi* and *Scenedesmus obliquus*.

2. The organic enrichment is essential for maintaining continued fertility of *D. magna*.

3. The organic enrichment can be replaced by the addition of pantothenic acid to vitamin B_{12} and thiamine without lowering the fertility of *D. magna*.

4. The organic enrichment (or the vitamin mixture) does not act directly on *D. magna* but *via* the algae by changing their nutritional value for *Daphina*.

5. Addition of vitamins to the medium in which the algal food is grown with crustacea may allow continuous cultures of herbivorous crustacea which are considered difficult to grow.

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SENSITIVITY OF ECHOLOCATION IN CAVE SWIFTLETS

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The specializations of bats of the suborder Microchiroptera for echolocation have naturally suggested that nocturnal or cave dwelling birds might orient themselves in the same way. Numerous preliminary tests of several species (mostly unpublished) have yielded negative results, and only two avian genera have been clearly demonstrated to use echolocation. These are the oilbirds of South America, *Steatornis caripensis* (Griffin, 1954), and certain species of the genus *Collocalia*, the cave swiftlets of Southeast Asia (Griffin, 1958; Novick, 1959; Medway, 1959, 1967). The orientation sounds of both species are brief audible clicks lasting a few milliseconds. They tend to have a peak of energy between two and eight kHz, but also a very broad acoustic spectrum extending to ultrasonic frequencies. The clicks are emitted primarily in dim light or darkness and increase in repetition rate like those of bats and cetaceans when difficult orientation problems arise. A very similar sort of echolocation is practiced by bats of the genus *Rousettus*, the only known case in the suborder Megachiroptera.

Although oilbirds and swiftlets can certainly avoid large obstacles by echolocation, there is very little evidence concerning their proficiency, and we know almost nothing about the minimum size of object that can be detected. In contrast considerable data on this point are available from bats of the suborder Microchiroptera (Griffin, 1958; Suthers, 1965, 1967; Schnitzler, 1966). Griffin, Novick, and Kornfield (1958) measured the size of cylindrical obstacles that could be detected by a single individual *Rousettus aegypticus*. With sufficient practice this animal could detect wires as small as 0.46 millimeter diameter at distinctly above the chance level. *Rousettus* uses as orientation sounds audible clicks that are similar to those of *Collocalia* and *Steatornis*, but in view of evidence that most small mammals can hear well at ultrasonic frequencies (Ralls, 1965) it is difficult to ascertain which part of the broad frequency spectrum emitted by *Rousettus* generates the echoes by which it detects small obstacles. Medway (1967) studied the ability of *Collocalia fuciphaga* to avoid vertical wooden rods one centimeter square spaced 15 cm apart. These birds with a wingspan of about 27 cm showed no ability to avoid these obstacles when first encountered in darkness. But they maneuvered between them almost perfectly in the light. Their performance in the dark improved on successive flights, but the experiment did not demonstrate how much of this improvement resulted from learning the positions of the obstacles and how much from echolocation. The many species of *Collocalia* appear to differ widely in their use of dark caves and their reliance on echolocation.

During the 1969 Alpha Helix expedition to New Guinea we studied the orientation sounds of *Collocalia vanikorensis granti*, and carried out preliminary experiments that indicate the approximate threshold size of cylindrical obstacles detectable by echolocation. This species nests in a totally dark chamber of a cave near Amele, south of Madang, which we visited twice in June, 1969, for preliminary observations. Local opposition of a quasi-religious nature prevented extensive studies of this colony, but through the kindness of Dr. Kiro Kikkawa three birds of the same species were obtained in mist-netting operations near Omuru within a few km of this cave.

METHODS

The obstacle avoidance tests were conducted in a dark chamber constructed from a double layer of heavy, opaque, black plastic. This was shaded and sheltered from rain by a fly of the same material below a thatched roof. A small air conditioner was used to maintain a temperature of approximately 26–28° C. The tests were conducted both in the daytime and after dark, but the chamber was sufficiently light-tight that even in bright sunlight, and when we were fully dark adapted, we could not see large white objects. All test obstacles were small wires or rods extending vertically from floor to ceiling and spaced 40 cm apart horizontally. With the lights off they were quite invisible. The chamber was 4.9 meters long, 2.9 meters wide and 2.1 meters high with its long axis approximately east-west. Near the center, 2.3 meters from the west end, a wooden frame around the walls, floor, and ceiling reduced the height to 1.86 meters. On this frame we mounted a series of cords and pulleys from which obstacles were mounted and shifted horizontally in position while maintaining their horizontal spacing. The obstacles mounted on this frame will be referred to below as the middle obstacles. Three additional rows of vertical obstacles were hung from hooks in the ceiling at 1.5, 2.4, and 3.9 meters from the west end of the chamber. These could also be shifted between the fixed positions of hooks in the ceiling, but this type of shifting was less convenient and was carried out less often. The obstacles in the middle plane were fastened by rubber bands at their tops and bottoms while those in the other three rows were held straight by light weights. The swiftlets almost always flew at least 30 cm below the ceiling and at an even greater distance above the floor, both in the light —where they could easily be seen—and in darkness where we could locate them by their audible orientation sounds. All trials considered below consisted of flights through the obstacle planes at 30 cm or more from walls, or ceiling. The smallest obstacles tested were supported by attachments of the same size as the larger obstacles. In view of the poor performance with the smallest obstacles tested we did not take stringent precautions against the possibility that the echolocation achieved by these birds was based on echoes from the weights stretching the obstacles at the bottoms of three rows, or the attachments of the ceiling.

Cave swiftlets are delicate birds which are difficult to maintain in captivity in good physical condition for more than a day or two, even though they were force-fed food mixtures suitable for insectivorous birds. All experiments considered below involved swiftlets that had been in captivity only one or two days and appeared approximately as vigorous and adept at avoiding obstacles as when first brought into the flight chamber. All data from these four birds during this period are included in Table I except for times when they would, after several minutes of flight, temporarily appear weak or refuse to fly the length of the flight chamber. One of us held each bird in the hand roughly 30–40 cm below the ceiling and released it only after the lights were extinguished. In most cases one of us watched with the infrared viewer from the opposite end of the flight chamber in the hope of observing the bird's flight path. This instrument afforded a clear view of the moving bird in only a minority of the flights, but careful listening to the orientation sounds sufficed to show the bird's approximate flight path, and as soon as it had passed through one to four barrier planes we switched on our flashlights and inspected the obstacles immediately to detect vibration or other motion. Unfortunately the sound of contact with obstacles could not be used, as we have commonly done with bats, because it was masked by the orientation sounds of the birds. Since no test was begun until all obstacles had again come to rest, and since even a light brush of a swiftlet's wing set the obstacles in evident motion, we feel confident that we detected most contacts with obstacles.

TABLE I

Obstacle avoidance scores of Collocalia vanikorensis granti *flying through an array of vertical wires and rods. N indicates number of trials, % M per cent misses. All flights in darkness or with the bird blindfolded. The flights marked "Shifted" were those immediately after the obstacles had been moved horizontally by several centimeters.*

Bird No.	Date	2 mm plastic covered wires		6.3 mm iron rods		8 mm plastic tubing	
		N	% *M*	*N*	% *M*	*N*	% *M*
1	23 July	20	50%				
	23 July (blindfolded)	56	46%			16	63%
2	23 July	82	48%			20	80%
	24 July	26	42%	17	76%	20	70%
3	25 July	55	31%	31	74%	31	58%
4	29 July	68	40%	29	83%	30	77%
	29 July (shifted)	2	50%	12	92%	10	70%
Total July 24–29		151	37%	89	80%	91	68%
Total of all birds		309	43%	89	80%	127	68%

The positions of the obstacles, particularly those in the middle row, were shifted horizontally from time to time without any noticeable effect on the birds' success at avoiding them. An especial effort was made to do this with bird No. 4. It was enclosed in the cupped hands while obstacles were shifted and released only after the lights had been turned off. The performance of this bird was somewhat better during flights immediately after such a shift in obstacle position. It thus seems unlikely that learning of the position of obstacles (Griffin, 1958, pp. 162–167; Neuweiler and Moehres, 1967) had any appreciable effect in these experiments. The two millimeter obstacles were shifted less often than the larger ones, so that the birds had a better opportunity to learn their positions, but this did not eliminate the substantial difference in avoidance of the larger and smaller cylinders.

The orientation sounds of two swiftlets were recorded as they flew in the darkened chamber with a Roberts model 6000 stereo tape recorder and Electro Voice model 644 microphones. The frequency response of this system was limited by the microphone which was flat within about ±3 dB from 700 to 12,000 Hz and down about 10 dB at 17,000 Hz. Portions of this recording were displayed on an oscilloscope and photographed for further analysis. Spectrographs of a number of clicks were also made with a Kay Electric Co. model 6061B sound spectrograph.

RESULTS

Obstacle avoidance scores for all four birds under all conditions tested are included in Table I for the sake of completeness, but there do not seem to be any significant differences between birds or conditions. Consequently the overall averages provide the most reliable picture. All data are pooled as in the final line of the table, but the last three days are also listed separately because on those days all three birds were tested with all sizes of obstacle. The results are consistent in showing a large difference between 2 millimeter and 6.3 millimeter obstacles and a somewhat poorer performance with the 8 millimeter plastic tubing. The flight paths of these birds were too variable and insufficiently well observed to allow any accurate calculation of chance scores, but the poorest performers registered about 30 to 40% misses. When we watched the birds with the infrared viewer our impression was that the two larger sizes of obstacles were avoided by turns and dodging maneuvers. But the resolution of this instrument under the conditions of our experiments was too poor to justify much confidence in this observation.

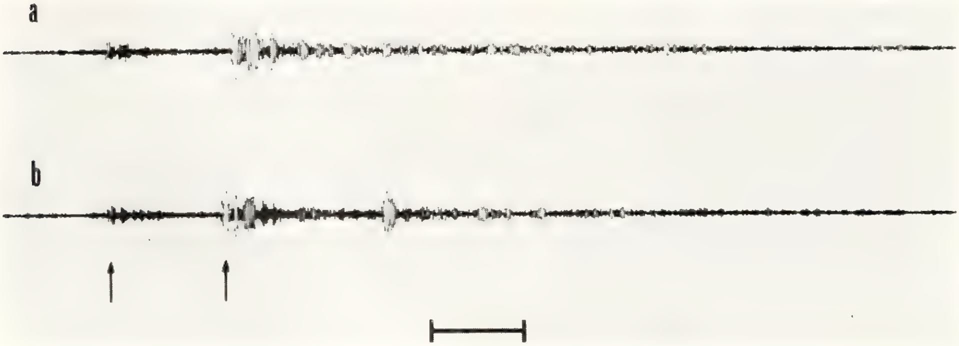

FIGURE 1. Oscillographic display of clicks emitted by *Collocalia vanikorensis;* (a) most commonly emitted type consisting of double click; (b) less common type composed of three high amplitude portions. Arrows indicate two initial high amplitude portions of envelope which are responsible for double vertical bars on sonagrams in Figure 2a. Bar equals 10 msec.

The poorer performance with 8 millimeter plastic tubing than with 6.3 millimeter iron rods is somewhat puzzling, since the echoes from the larger cylinders must have been more intense. Our impression was that motion of the iron rods after a light touch was more difficult to observe and more quickly damped out. Thus we feel the most likely explanation for the drop from 80 to 68% misses was that we failed to detect motion of the 6.3 mm rods after some light touches.

Each orientation sound typically consisted of a moderate amplitude peak a few milliseconds long, followed after several milliseconds by an appreciably higher intensity peak about 4 to 8 msec in duration (Fig. 1a). This second high amplitude peak continued as a gradually attenuated complex waveform which disappeared into the noise level of the recording after about 30 to 50 msec. Much of this terminal portion was probably due to echoes from the chamber walls, floor or ceiling. The double nature of each click was clearly audible when the tape recording was reproduced at one-eighth its original speed. Occasional pulses also showed a third high amplitude peak (Fig. 1b). Click intervals (measured as the time between the maximum amplitude portions of successive clicks, rather than the silent intervals between them) for 477 clicks comprising several flights averaged

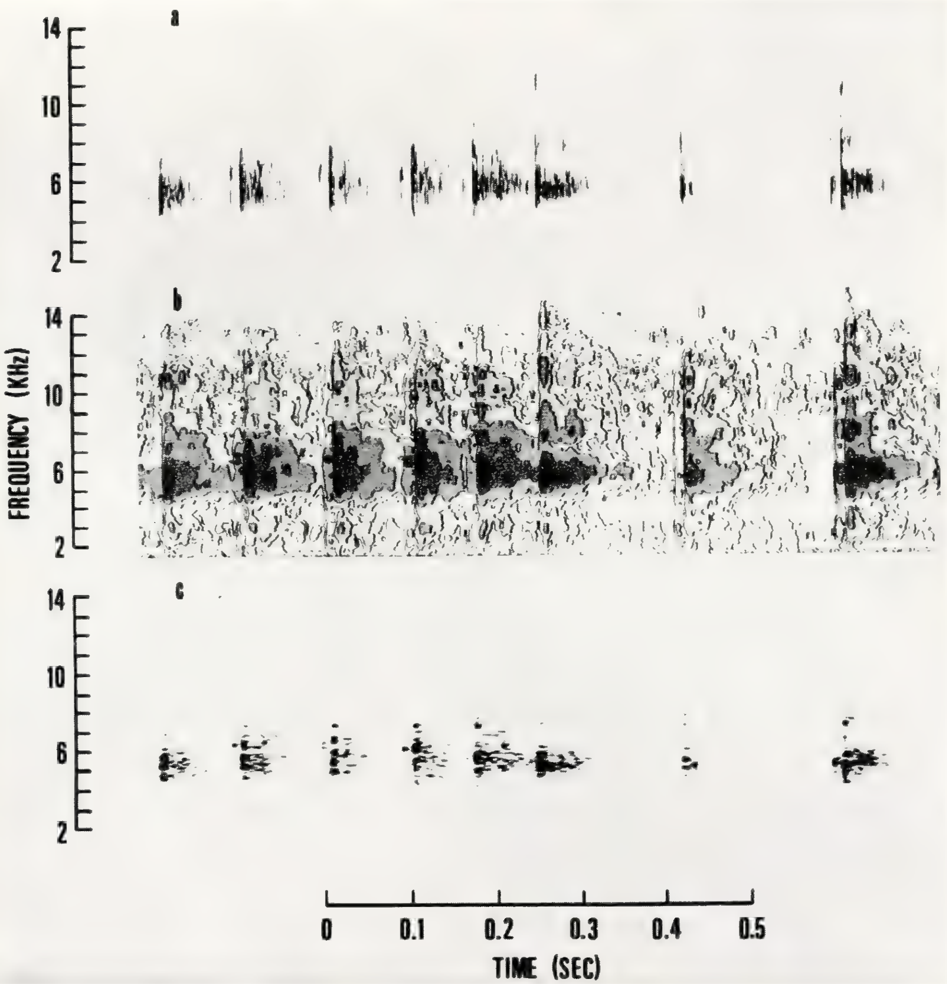

FIGURE 2. Sound spectrographs of eight successive orientation clicks of a single *C. vanikorensis* flying in the dark. Approximate sensitivity of recording system uniform within ±3 dB from 0.7 to 12 kHz and down 10 dB at 17 kHz; (a) wide band width display (300 cps at 3 dB down) showing temporal relationships; (b) intensity contour plot indicating relative intensity by 6 dB steps between contours. Darkest contours represent most intense sounds; (c) narrow band width display (45 cps at 3 dB down) giving better frequency resolution but blurring temporal relationships and losing some lower intensity portions.

116 msec with a minimum of 48 msec and a maximum of 358 msec, corresponding to repetition rates of about 3 and 20 clicks per second, respectively.

The double nature of the clicks is readily visible in the spectrograms (Fig. 2) which also indicate the distribution of sound energy in each click. It is evident that clicks of *C. vanikorensis* have a wide frequency spectrum most of which appears to lie within the human audible range. The highest intensity sound occurs between 4.5 and 7.5 kHz, but as in all impulsive clicks there is appreciable sound energy at higher frequencies. A few clicks showed small amounts of acoustic energy as high as 16 kHz, the upper frequency limit of the spectrogram, but this is uncommon. Very faint low frequency portions of the clicks extend downward to about 2 kHz.

DISCUSSION

Medway (1959) reported most of the acoustic energy of clicks emitted by *Collocalia maxima lowi* occurred between 2 and 4.5 kHz with very little energy below 1.5 or above 5.5 kHz. Novick (1959) found the principal frequency of *Collocalia brevirostris unicolor* to lie between 4 and 5 kHz but noted many overtones. These apparent interspecific differences may in part reflect different sensitivities of the various tape recorders and microphones to high frequencies. It does however appear that clicks produced by *C. vanikorensis* contain relatively more high frequency components than do those of other *Collocalia* thus far studied. The presence of these high frequencies could theoretically improve the bird's ability to detect echoes from small objects. It is interesting to note that Medway (1967) found *C. fuciphaga*, which emits clicks with frequencies principally in the range between 1.5 and 4.5 kHz, unable to echolocate a barrier of wooden rods 1 cm^2 in cross section, whereas our data indicate *C. vanikorensis* regularly echolocates iron rods 6.3 mm in diameter.

While it would clearly be desirable to obtain considerably more extensive data involving more birds, a wider range of obstacle sizes and spacings, and more trials, these results are of interest because no previous investigations have included even such approximate determinations of the threshold sizes of obstacles which *Collocalia* can detect by echolocation. Under more or less favorable conditions the threshold size of obstacles may vary to some extent, but it seems unlikely that it would be far outside the interval between 2 and 6 millimeter diameter cylindrical obstacles.

Although the auditory sensitivity of *Collocalia* has not been studied directly, the extensive data on other birds (Schwartzkopff, 1968; Konishi, 1969) suggest that they are not likely to have high auditory sensitivity extending above 20 kHz. In the absence of direct evidence, it seems most likely that *Collocalia*, like all other birds that have been adequately studied, have a frequency range of hearing roughly comparable to our own. It also seems likely, though not absolutely certain, that *C. vanikorensis* employs orientation sounds without useful components above the range of human hearing. If so, the wavelengths of sound used by these birds for echolocation are also available to blind men attempting echolocation based on audible sounds. This implication of the still very poorly understood acuity of echolocation in *Collocalia* justifies further and more intensive study of the questions discussed in this paper.

The support of the National Science Foundation is gratefully acknowledged, including both the 1969 ALPHA HELIX Expedition, and grant GB 7155 to the New York Zoological Society.

SUMMARY

The acuity of echolocation in *Collocalia vanikorensis granti* was studied by measuring the success of freshly captured birds in avoiding cylindrical obstacles in total darkness. The percentage of misses varied from approximately 40% with 2 millimeter insulated wires to 80% with 6.3 millimeter iron rods and 68% with 8 millimeter plastic tubing. The orientation sounds used for echolocation are clicks with the major energy between 4.5 and 7.5 kHz. Although the frequency range of hearing in these birds has not yet been measured, it seems probable that they can echolocate obstacles as small as 6 millimeter diameter rods by means of frequencies within the range of human hearing.

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THE ROLE OF AMOEBOCYTES IN THE REGENERATION OF SHELL IN THE LAND PULMONATE, *EUPLECTA INDICA* (PFIEFFER)

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The presence of amoebocytes at the site of shell regeneration in gastropods has been observed by several workers. But opinions and observations differ as to the manner in which the amoebocytes participate in the repair process. Durning (1957) states that in *Helix aspersa* amoebocytes appear along cracks in the regenerating membrane due to irritation and he dismisses them as unconnected to shell repair. McGee-Russell (1954) also noted in regenerating snails the presence of some cells in the extrapallial fluid that originated from mantle epithelium but he did not assign them any role in the repair process. Abolins-Krogis (1963, 1968) opines that during shell repair, amoebocytes transfer large amounts of repairing materials like proteins and calcium from the yellow body cells and digestive gland cells to the actual site of shell repair, liberate it there and withdraw from the site. But some of the cells become accidentally trapped within the calcifying plates of the shell. To complicate matters further, studies of Kapur and Gibson (1967) on the normal development of shell in *Helisoma duryi eudiscus* point to the appearance of amoebocytes on the ostracum of the shell in the juvenile snails and that later these form multinucleate organic plates. These have been suggested to be directly involved in the calcification of the adult shell.

With such divergent views on the role of amoebocytes in the repairing as well as normal shells, the controversy is wide open for more studies. Also since studies on shell repair in gastropods have almost exclusively been on a single land snail, *Helix*, it was decided that new investigations should be carried out in other genera of land pulmonates. The present communication deals with the regeneration of shell in *Euplecta indica* (Pfeiffer), a terrestrial pulmonate from Chandigarh, India.

MATERIALS AND METHODS

Specimens of *Euplecta indica* were collected from a garden in Chandigarh. They were maintained in a terrarium at 21° C and were fed on cabbage leaves. Seven groups of thirty snails each were used in this study. A hole, about 3 to 4 mm in diameter, was made in the shell of each snail, some distance behind the shell aperture. It was then covered with a piece of glass coverslip, using warm paraffin wax for pasting. The snails deposited the regenerating shell material on the undersurface of the coverslips. At ten different intervals, ranging from 1 hour to 108 hours, the coverslip bearing the regenerate material was removed from the shell, placed in the required fixative and processed accordingly. Regenerate materials obtained thus at different intervals from each group of 30 snails were stained with the following techniques: group 1, haematoxylin and eosin; group 2, toluidine blue;

group 3, alcian blue-Delafield haematoxylin; group 4, mercuric bromophenol blue (Hg-BPB); group 5, periodic acid-Schiff (PAS) with and without diastase digestion; group 6, Sudan black B and group 7, Feulgen test. Regenerate samples from 1 to 4 and 7 were fixed in 10% neutral formaline, from group 5 in Bouin's fluid and from group 6 in formol-calcium. Some neutral formalin-fixed samples of shell regenerate were tested for the presence of calcium, using the von Kossa technique. In group 3, Delafield haematoxylin was used as a nuclear counterstain instead of the recommended neutral red because batches of this dye then available were not compatible with the alcian blue technique. All the histochemical techniques were carried out as described by Pearse (1961). The regenerate-bearing pieces of coverslip after staining, dehydration and clearing were mounted as whole mounts in Canada balsam, with the regenerate-bearing surface facing upwards. Samples from group 6 were similarly mounted in glycerine jelly.

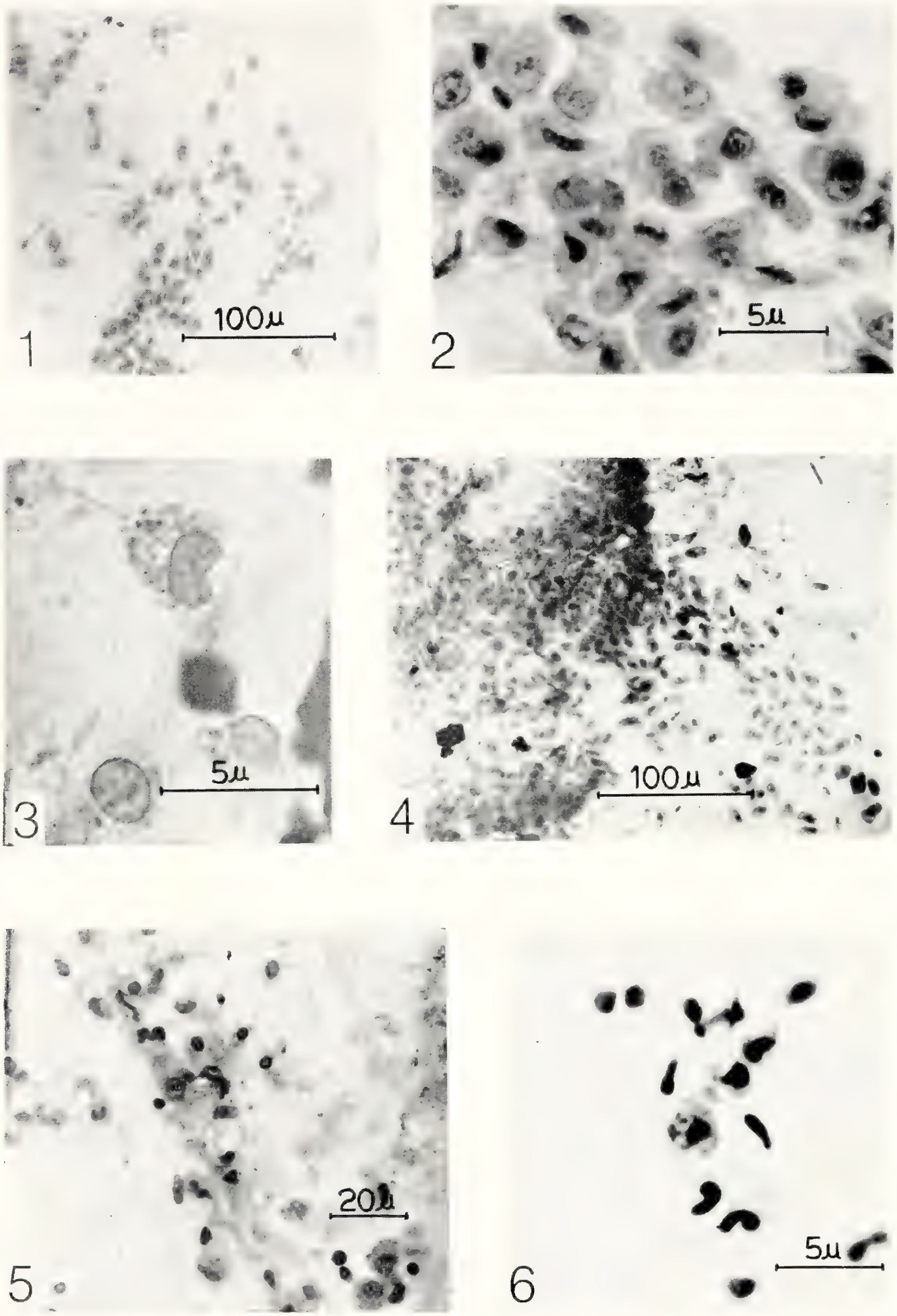
It was observed during this study that given everything equal, including size of animals, their time of collection and feeding, the rate of shell repair varied between individuals. The timings given are not rigid invariables, but represent the average conditions seen in the greatest number of individuals in all the groups used in this study.

OBSERVATIONS

The process of shell repair is initiated by the deposition of a glistening, translucent regeneration membrane, one hour after shell damage. This membrane stains uniformly blue with haematoxylin, is uniformly PAS positive with and without diastase digestion, is slightly metachromatic in toluidine blue, stains bluish-green in alcian blue, gives a positive test with Hg-BPB and is also weakly sudanophil. No localization of staining was observed by any of the above techniques, as the regeneration membrane is homogeneous and structureless. The above mentioned tests show the presence of neutral mucopolysaccharides, glycoproteins, mucoproteins, acid mucopolysaccharides, proteins and small amounts of lipids.

One hour after the start of regeneration, amoebocytes suddenly make their appearance on the regenerating membrane, continue to increase in number until at six hours their number reaches the highest peak (Fig. 1). Two types of amoebocytes were observed and we designate them as the a-type and the b-type. Nuclei of a-type cells are deeply basophilic with a compact coarsely granular meshwork of chromatin. Nuclei of b-type cells are vesicular, with scattered chromatin granules interlinked by fine fibrils. The nucleo-cytoplasmic ratio of the two kinds of cells also differs: nuclei of a-type cells occupy a third or a quarter of the total cell volume whereas nuclei of the b-type cells occupy more than half the extent of the cell (Fig. 2). Further, the a-type amoebocytes are about half the size of the b-type amoebocytes, but occasionally acquire the full size of the b-type amoebocytes. Occasionally the nuclei of some a-type amoebocytes possess U, V, J or dumbbell shapes. Possibly the nuclei of these cells are undergoing necrosis (Fig. 6).

The nuclei of both types of cells showed the usual basophilia with haematoxylin and toluidine blue. Besides, the chromatin granules in both types of nuclei show metachromasia with toluidine blue and also stain positively with alcian blue. The general cytoplasm of both cells is eosinophilic toluidine blue positive and alcian blue positive. The cytoplasmic granules of the a-type amoebocytes show metachromasia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Figures 1–6.

with toluidine blue. Calcium is present in the cytoplasm of both types of amoebocytes in the form of granules (Fig. 3). Nuclei of both cell-types give a positive test with Hg-BPB but the nucleus of the a-type does so more intensely, while the nucleoplasm of the b-type remains unstained. The cytoplasm of both cell-types is also positive to Hg-BPB but in the b-type cells, a narrow perinuclear zone remains unstained. Nuclei of both cell-types are negative to Sudan black B staining but their cytoplasm is diffusely positive. Some sudanophilic granules are present in the perinuclear zone which as stated above, gives no response to Hg-BPB staining. Nuclei of both cell-types are Feulgen positive.

The preceding observations relate to the amoebocytes that have just arrived at the regeneration site. But soon, about an hour after their arrival, the amoebocytes cluster and start losing their identity (Fig. 4). It appears that their outer cell membranes rupture and their cytoplasm precipitates, leaving their nuclei in a free, denuded state (Fig. 5). Such patches of precipitated cell material exhibit the same staining reactions as in the intact amoebocytes. They give positive results for neutral mucopolysaccharides, glycoproteins, mucoproteins, acid mucopolysaccharides, proteins and lipids. The free nuclei are seen as long as 83 hours after the precipitation of the amoebocytes. However, gradually, these nuclei become pycnotic, diminish in size and finally disintegrate. Such disintegrated nuclear material along with the precipitated cytoplasm is still Feulgen positive. The a-type cells with U, V, J and dumbbell-shaped nuclei also behave in the same manner (Fig. 6).

The site of repair now has a regeneration membrane bearing large numbers of intact amoebocytes and also patches of precipitated cells which had arrived earlier. In addition, numerous fine tracts of acid mucopolysaccharides are observed. These are possibly derived from the secretion products present in the extrapallial fluid which is constantly bathing the regenerating material.

The first indication of the initiation of the calcifying process of the regenerate is given by the appearance of small crystals of calcium carbonate on top of or along the precipitated cytoplasm of the amoebocytes and the fine tracts of acid mucoploysaccharides. These crystals are in spheritic double-fan shaped arrays (Fig. 7). Thus the materials contributed by the amoebocytes serve as the calcifying substratum. As crystallization proceeds, a steady decline in the metachromasia of the organic substratum is noted. The regeneration plate thus comes to have several calcifying tracts showing crystallization of calcium carbonate, coincidental with the tracts of cellular precipitation. The process of arrival of amoebocytes and their precipitation is a continued one, occurring simultaneously with crystal formation from this point onwards. Thus the precipitation of the amoebocytes contributes to

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FIGURE 1. Two-hour regenerate: amoebocytes on the regeneration membrane; toluidine blue.

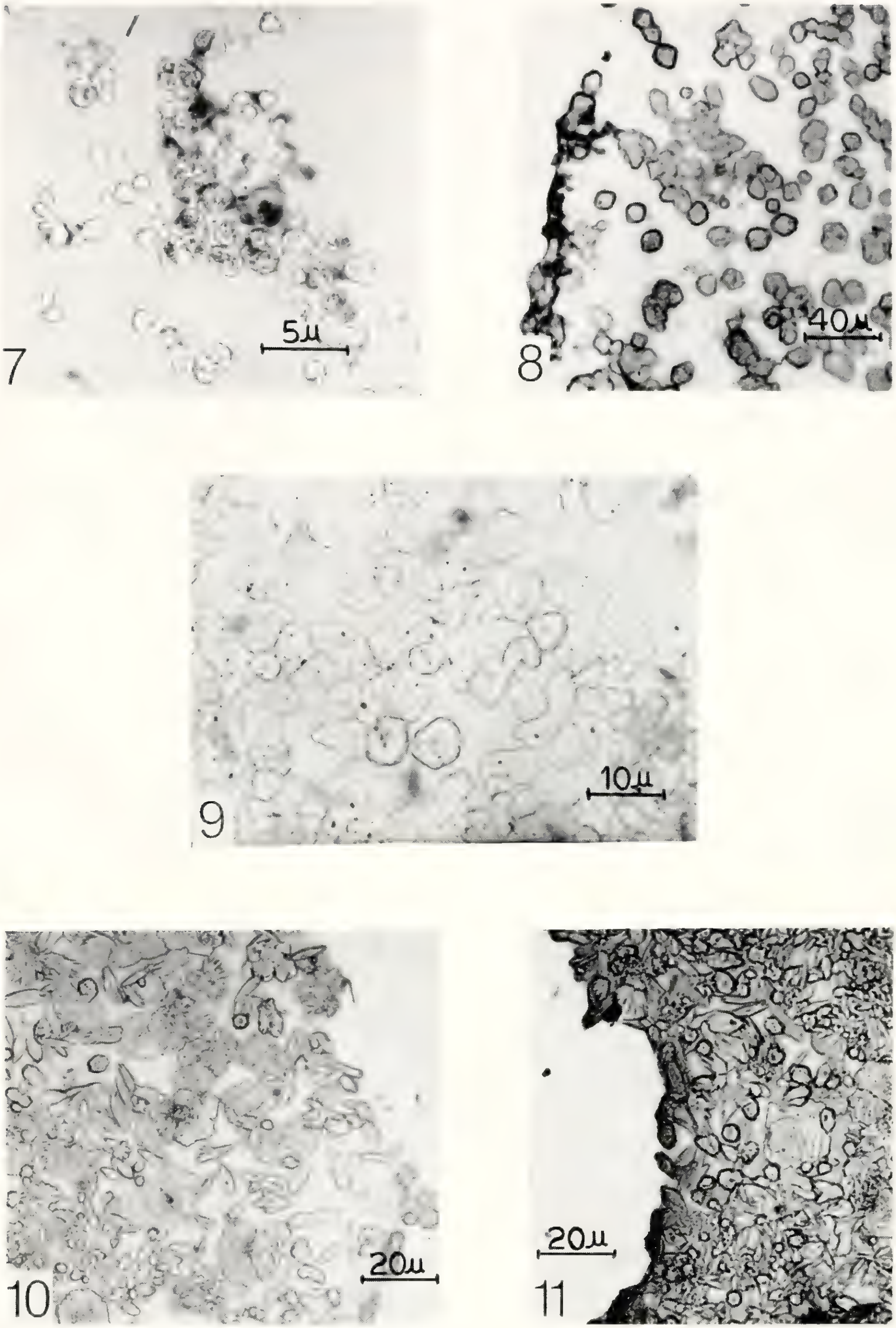
FIGURE 2. Six-hour regenerate: a- and b-type amoebocytes. Note the difference in the nuclei of the two cell types; toluidine blue.

FIGURE 3. Four-hour regenerate: note the calcium granules in the cytoplasm of the amoebocytes; von Kossa-neutral red.

FIGURE 4. Six-hour regenerate: intact cells at right, precipitated cells on the left of the picture; toluidine blue.

FIGURE 5. Six-hour regenerate: free nuclei lying in the precipitated cytoplasm; alcian blue-Delafield haematoxylin.

FIGURE 6. Six-hour regenerate: free pycnotic nuclei of various shapes; one intact b-type amoebocyte can be seen; toluidine blue.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Figures 7–11.

the formation of the organic ostracum upon which the growth of the mineral fraction of the shell proceeds. The growing calcium carbonate crystals soon acquire a tabular form (Fig. 8).

As these crystals grow larger, they meet adjacent growing tabulae at their peripheries and coalesce with them (Fig. 9). Small acicular crystals appear in parallel and in spheritic aggregates in the spaces between such intergrowing tabulae (Fig. 10). A second layer of crystals is initiated on top of the first layer even before the latter is completed. Thus the regenerate grows in extent as well as in thickness at the same time.

At 24 hours the regenerate consists of an almost complete sheet of tabulae, with most of the intervening spaces filled up by parallel and spheritic aggregates of crystals (Fig. 11). New batches of amoebocytes continue to arrive and precipitate, the only difference now being that whereas the first batches arrived and precipitated on the regeneration membrane, the later arrivals precipitate upon the crystalline layers. Successive layers of crystals are laid down until in about a week's time the regenerate approaches the original shell in thickness.

In surface view the regenerate appears as a thick conglomerate of crystals. It actually consists of several indistinctly separated crystalline sheets, each of which may be seen in a different plane of focus at the edges.

The fully regenerated shell is intimately fused with the original shell and cannot be easily separated from it. The regenerate lacks a periostracum, which is present in the normal shell. It also differs from the latter in its random arrangement of crystals and in its partly cellular origin, of which the normal shell presents no evidence.

Discussion

It has been observed in this study that within an hour of shell damage, a homogeneous, thin, translucent membrane appears over the damage area of the shell. This has probably come about by the precipitation of secreted organic substances present in the extra-pallial fluid. To this extent shell regeneration is a physicochemical process. But we cannot agree with workers like Manigault (1933) and McGee-Russell (1954) on *Helix* that the entire regeneration of shell is brought about in this manner. On the other hand observations of Wagge (1951) also on *Helix* that the regeneration membrane is formed exclusively by the amoebocytes which arrive at the site to deliver proteins and calcium are not tenable here. What we find is that once the precipitated membrane is formed at the beginning of regeneration, it acts as a scaffolding for the amoebocytes to perform their role in regeneration.

Figure 7. Seven-hour regenerate: initial double-fan shaped and spheritic crystal aggregates in association with the cellular precipitate and two free pycnotic nuclei; toluidine blue.

Figure 8. Nine-hour regenerate: growing tabloid crystals on the organic ostracum.

Figure 9. Ten-hour regenerate: intergrowth of tabulae. Note coalescence at the margins of the crystals.

Figure 10. Twelve-hour regenerate: parallel and spheritic crystal aggregates appearing in the spaces between the tubulae.

Figure 11. Twenty-four-hour regenerate: note that the spaces between the tabulae are nearly filled by the parallel and spheritic aggregates of crystals.

Further it is observed that an hour after the start of shell regeneration, large numbers of amoebocytes arrive at the newly formed regeneration membrane. But unlike observations of Wagge (1951) it is found that these cells do not merely deliver their payloads and then withdraw from the membrane. Instead, it appears that the amoebocytes lose their identity, their outer cell membranes become disorganized and their cytoplasm precipitates, leaving free nuclei. In fact the amoebocytes arrive in such large numbers that the original regeneration membrane is no longer seen. Histochemical tests on the intact and disorganized cells reveal the presence of large amounts of protein, PAS positive mucopolysaccharides and acid mucopolysaccharides. These substances are necessary components of most calcifying matrices in animals. Besides, calcium granules were also detected in the cytoplasm of the amoebocytes. These findings are in agreement with those of Abolins-Krogis on *Helix* (1960, 1963 and 1968) that amoebocytes carry histochemical substances necessary for calcification from different organs of the body to the site of regeneration. We further find that not only are these substances transferred to the regeneration site, but also that the cytoplasm of the amoebocytes contributes predominantly to the formation of the organic ostracum on which the calcification processes are initiated.

The calcification of the regenerate is initiated by the appearance of small double-fan shaped spheritic aggregates of crystals. These initial crystals have also been observed by Abolins-Krogis (1968) in her studies on *Helix,* although she invests their structure with a large amount of organic content.

The metachromasia noticed prior to the beginning of crystallization is seen to recede in the later stages of calcification. This may be indicative of the binding of calcium by acid radicles on the acid mucopolysaccharide molecules (Tanaka and Hatano, 1955; Horiguchi, 1956; Kado, 1960) which are in association with the protein matrix. As the tabloid crystals grow large, they fuse with adjacent crystals and tend to form a continuous sheet. New batches of amoebocytes continue to arrive and settle over the crystalline sheets and thin films of organic material which is probably continually precipitated from the extrapallial fluid. Soon the amoebocytes become disorganized, spill over their contents and start calcification of another crystalline sheet once again.

Although no cellular involvement is noticeable in the structure of the adult shell of *Euplecta,* the involvement of amoebocytes in the calcification of developing and adult shell is known from the studies of Kapur and Gibson (1967) and Kapur and Bansal (in preparation). In fact, Kapur and Gibson found precipitation of the newly arrived amoebocytes on the protoconch and subsequent initiation of calcification in *Helisoma duryi eudiscus.* The disruption of amoebocytes at the site of shell regeneration reminds us of a rather similar behavior of leucocytes in wound healing in vertebrates.

The authors are grateful to Professor G. P. Sharma, Head of the Department of Zoology, Panjab University, for encouragement and for providing the necessary laboratory facilities during the course of this work.

SUMMARY

An hour after the damage of the shell in *Euplecta indica* (Pfieffer) a thin, structureless, basophilic regeneration membrane appears. Thereafter, a large number of amoebocytes precipitate on this membrane. Soon these break up and contribute their cytoplasmic contents to the regenerate. This is positive for glycoproteins, mucoproteins, neutral mucopolysaccharides, acid mucopolysaccharides, proteins, calcium and some lipids. Also, tracts of acid mucopolysaccharides precipitate from the extrapallial fluid and are deposited on the regenerate.

Calcification is initiated by the appearance of seed crystals of calcium carbonate on top of or along areas of cellular precipitation. This leads to a progressive decrease in metachromasia. The crystals grow into large tabular forms, coalesce with the adjacent tabulae and form a calcified sheet of regenerated material. The spaces between the tabulae are later packed by small crystal aggregates of various shapes. More and more amoebocytes continue to arrive, settle on the calcified sheets along with precipitated organic matter from the extrapallial fluid, break up and repeat the calcifying process until a regenerate of the same thickness as the normal shell is formed.

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"DISTANCE EFFECT" IN PIGEON ORIENTATION: AN EVALUATION

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In several papers, Matthews (1955, 1963) has reported that his pigeons exhibit poorer orientation when released at intermediate distances than when released at very short distances or at long distances. He interprets this "distance effect" as indicating that the pigeons' system of navigation is not sufficiently accurate to be used effectively at distances less than about 80 km. Matthews explains the accurate vanishing bearings of his pigeons at very short distances as probably being due to pilotage by familiar landmarks rather than to true navigation. He suggests that at intermediate distances the birds are too far from home to use landmarks but still too close to use accurately their bicoordinate navigation system, hence their poor homeward orientation.

Mittelstaedt (*in* Schmidt-Koenig, 1963a, 1963b) examined orientation as a function of distance in terms of the homeward component (*i.e.,* the component of the mean vanishing vector that is in the homeward direction), but the results of his analysis of the initial orientation of naive pigeons in Germany were inconclusive. An analysis by Schmidt-Koenig (1965, page 244) of the orientation of Matthews' experienced pigeons in England provided no convincing evidence of a distance effect, nor was convincing support for such an effect obtained in an elaborate series of releases conducted by Schmidt-Koenig (1963) in North Carolina. Later, however, Schmidt-Koenig conducted extensive tests of the distance effect in experienced pigeons flown in both North Carolina (1964, 1966) and Germany (1968), and published results that seemed to support Matthews' ideas. He found high values of the homeward component at distances less than about 19 km and greater than about 96 km; between these distances there was a zone of poor orientation. Wallraff (1967) reported similar results with untrained pigeons released on their first homing flights in Germany.

If the distance effect is a general characteristic of pigeon homing, it has important implications for the nature of the navigation system used by these birds. It would be consistent, for example, with a system such as the sun-arc hypothesis of Matthews (1953), which would be effective at short distances only if the birds could determine the sun's position with far greater precision than most workers have believed possible. Matthews (1968) has recently relied heavily on the distance effect in interpreting much of the published data on pigeon homing.

Because of the theoretical importance of the distance effect if it really exists, and because I have doubted its general occurrence in view of the very good homeward orientation regularly exhibited by both our untrained (Keeton and Gobert, 1970) and our experienced (Keeon, 1969) pigeons at the intermediate distances where orientation should be poorest, my colleagues and I conducted, during 1968 and 1969, an extensive series of test releases to determine whether any distance effect is evident in the performance of our pigeons.

METHODS

Test releases were performed at various distances north, east, south, and west of the Cornell pigeon lofts at Ithaca, New York. More particularly, the release sites were: North—5.9, 10.0, 26.8, 49.0, 84.6, 143.3, 200.3 km; East—5.7, 14.9, 33.5, 60.9, 91.1, 129.8, 164.2, 204.2 km; South—5.4, 12.1, 20.5, 32.6, 47.3, 49.5, 84.5, 124.6, 188.3 km; West—9.0, 17.8, 33.9, 47.2, 70.3, 119.9, 155.9, 177.7 km. At all but the most distant sites, a minimum of three test releases (usually more) were conducted, using different birds, and on different days. Except in 12 instances, at least 10 single-tossed birds (mean = 14.7) were used in each test release. Included in the analysis reported here are the results of a total of 172 test releases involving a total of 2525 single tosses of birds.

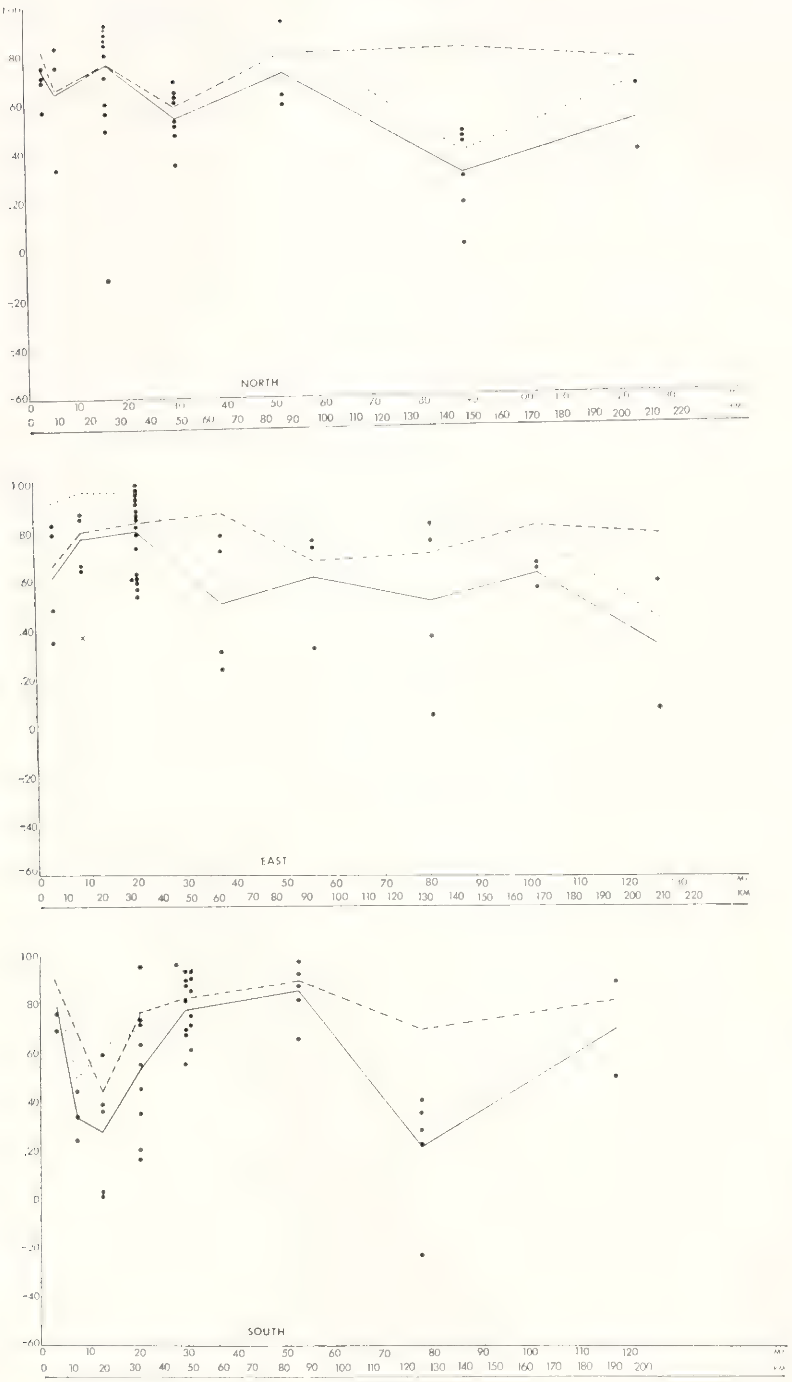
All birds used in the test releases had had previous homing experience from all four cardinal directions. For releases at 20 km or less, the birds' previous experience had included single-toss flights from distances this great or greater. For releases at more distant sites, the birds had previously made single-toss flights from distances at least as great as that of the next nearer site in the series.

In each test release, the birds were tossed individually from the hand, the directions in which they were pointed at the toss being randomized. Each bird was observed with 10 × 50 binoculars until it vanished from sight, and a compass bearing for the vanishing point was recorded to the nearest 5 degrees. The interval between toss and vanish was timed with a stop watch. No bird was released until the previous bird had been out of sight at least 5 minutes.

The circular mean (both direction and vector length) of the vanishing bearings of each release was calculated by vector analysis, following the procedure outlined by Batschelet (1965). Bearings were tested for randomness by means of the Rayleigh test (Batschelet, 1965). The homeward component was calculated as proposed by Mittelstaedt (*in* Schmidt-Koenig, 1963a, 1963b): $h = a \cdot \cos(\alpha - \beta)$, where a is the length of the mean vector, α is the mean direction, and β is the home direction. The homeward directional component was calculated by the equation: $d = \cos(\alpha - \beta)$. Values for both the homeward component and the homeward directional component may range from -1 to $+1$; values for the length of the mean vector may range from 0 to $+1$.

For each of the four directions, the homeward component (HC), homeward directional component (HDC), and length of mean vector (LMV) were plotted as a function of the distance of the release sites from the loft. In each case, the curves are based on the arithmetic means of the corresponding values obtained from all tests at each site.

Since it seemed possible that the length of the vanishing intervals (elapsed time between toss and vanish) might indicate something about the relative difficulty of orienting at different release sites, the mean vanishing interval for each release was calculated, and then the means of the means for all tests at each site were plotted as a function of distance.


100
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NORTH
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FIGURE 1, A–C.

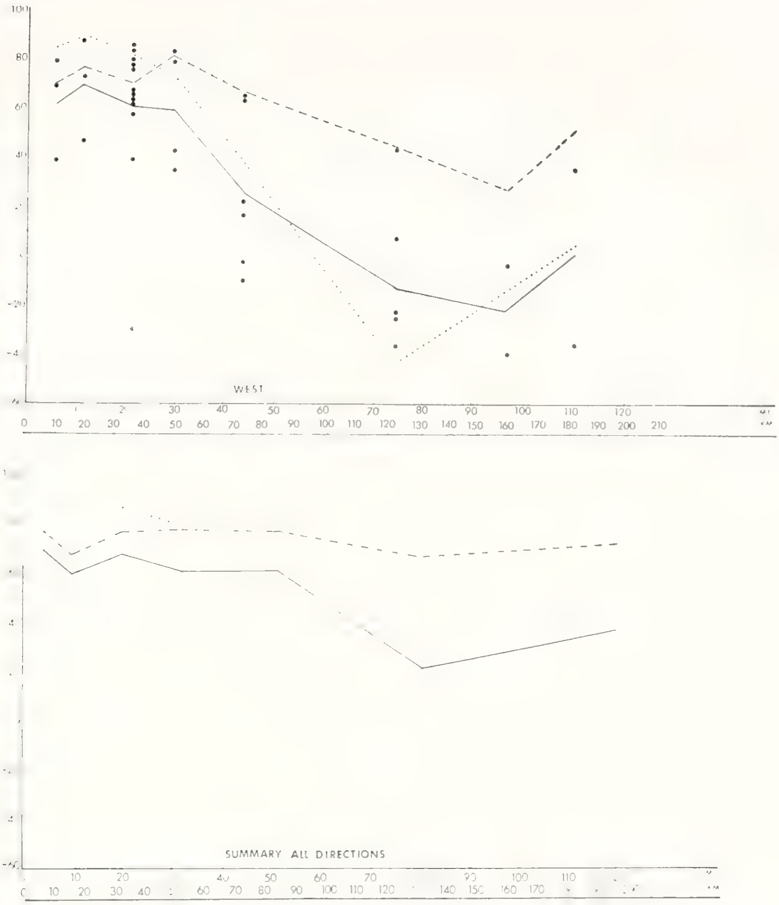

FIGURE 1. Graphs of the homeward component (solid curves), homeward directional component (dotted curves), and length of mean vector (dashed curves) plotted against distance of release from the home loft. The black dots indicate the values of the homeward component for the individual test releases; the curve connects the arithmtic means of the values for each site; A, releases from north of the loft; B, releases from the east; C, releases from the south; D, releases from the west; E, summary of all releases. The value indicated by X at 15.1 km in graph B is the mean homeward component for five releases at the alternate site discussed in the text.

RESULTS

Figures 1A, 1B, 1C, and 1D show the results of our releases from north, east, south, and west of the Cornell lofts, respectively. Figure 1E is a graph of the results of averaging the values from the four directions.

Not only do our graphs of the homeward component show little resemblance to those based on Schmidt-Koenig's data, but also they show few resemblances among themselves. There is no indication that the homeward component varies consistently as a function of distance in the manner suggested by Matthews, Schmidt-

Koenig, and Wallraff. More specifically, the poor orientation reported by those authors for intermediate distances is not seen—indeed the average of the homeward components for 27–34 km (17–21 miles) is 0.67, a remarkably high value; it is difficult to imagine that significantly better orientation could be obtained consistently at any distance.

That the good orientation at intermediate distances shown by the experienced birds used in the graphed releases is not simply a result of training is demonstrated by the equally good orientation at these distances regularly exhibited by our first-flight pigeons. Figure 2 shows examples of typical releases of such birds at roughly

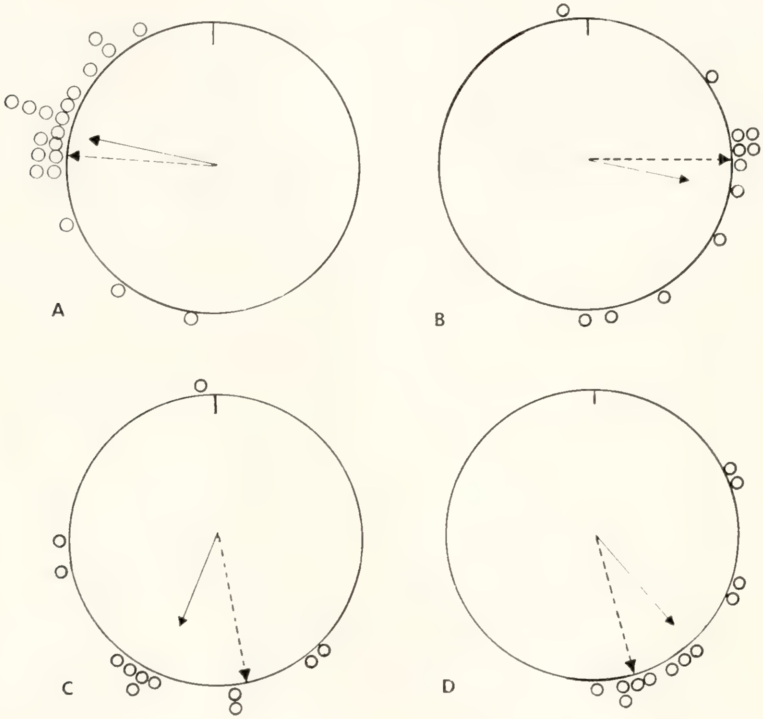

FIGURE 2. Examples of vanishing bearings of first-flight pigeons (ones never before taken away from the loft) released under sunny conditions: A, Harford, New York, 9.4 miles from the loft, April 25 and May 5, 1969, home bearing 276°, mean bearing 283° (nonrandom, $P < 0.0001$); B, Burdett, New York, 21.1 miles, October 23, 1968, home bearing 91°, mean bearing 101° (nonrandom, $P = 0.002$); C, near Locke, New York, 16.6 miles, October 23, 1968, home bearing 171°, mean bearing 205° (nonrandom, $P = 0.005$); D, Fleming, New York, 30.4 miles, December 12, 1968, home bearing 164°, mean bearing 139° (nonrandom, $P = 0.0007$). [In this figure and in Figures 4 and 5, the home direction is indicated by a dashed arrow, true north by a thin line at the top of the circle, and the mean vector by a solid arrow whose length is inversely proportional to the extent of scatter (the arrow would reach the periphery of the circle if there were no scatter, *i.e.*, if all the birds vanished in exactly the same direction, and the arrow would have 0 length if the vanishing bearings were uniformly scattered around the compass). Each symbol on the periphery of the large circle represents the vanishing bearing of one bird.]

16, 32, and 48 km (10, 20, and 30 miles); other examples have been published elsewhere (Keeton and Gobert, 1970).

Figure 3 shows mean vanishing intervals plotted against distance. The curves for the four cardinal directions show few resemblances among themselves. Except that for all directions vanishing intervals tend to be short at near (4–20 km) release points, there is no consistent relationship between vanishing interval and distance. In this regard, our results agree with those of Schmidt-Koenig (1966).

Discussion

In the published studies of distance effect, only the homeward component has been used as a measure of orientational performance. But the homeward component is not a particularly good measure since it is sensitive both to the deviation of the mean vector from the true home direction and to the extent of scatter of the bearings. Thus a homeward component of 0 could result from many fundamentally different distributions of the vanishing bearings, including two extreme distributions that are the opposites of each other—a circularly uniform distribution (*i.e.*, one with no mean vector) or a distribution in which all bearings deviate 90° in the same direction from home (*i.e.*, one as different from circular uniformity as a distribution can possibly be, but with the mean vector oriented at right angles to home). Clearly these distributions would have very different biological meanings, yet the homeward component permits no distinction between them.

Because of the desirability of distinguishing between the effects of mean directional error and the effects of scatter, the graphs in Figure 1 show, in addition to

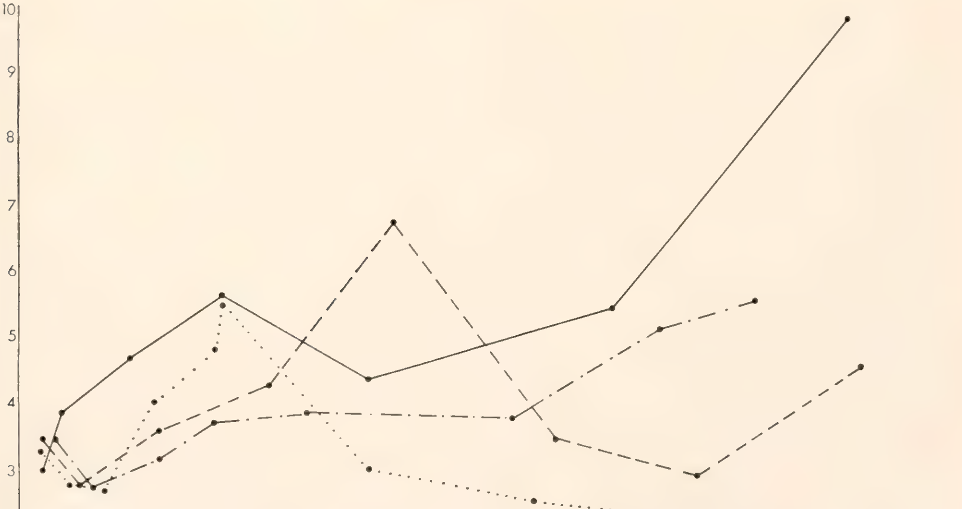

Figure 3. Graph of mean vanishing intervals (in minutes) plotted against distance of release from the home loft; North, solid curve; East, dashed curve; South, dotted curve; West, dot-dashed curve.

the homeward component, the homeward directional component and the length of the mean vector. The homeward directional component is a function only of the deviation of the mean vector from the home direction, and the length of the mean vector is a function only of the extent of scatter of the bearings. Hence these two indices give independent measures of the two variables that together determine the homeward component.

The figures indicate that the homeward directional component and the length of the mean vector vary independently. Thus, in Figure 1A it can be seen that the rises and falls of the homeward component curve at the shorter distances are due almost entirely to variations from site to site in the degree of scatter of the bearings, the accuracy of the mean bearings remaining nearly constant, whereas at the longer distances the shape of the curve is determined by variations in the accuracy of the mean bearings, the degree of scatter remaining nearly constant. In Figure 1B, the similar values for the homeward component at the release sites 61 km and 91 km from the loft obscure the fact that at the 61 km site the mean vector deviates considerably from the home direction (mean HDC = 0.56) but the scatter of the bearings is minimal (mean LMV = 0.87), whereas at the 91 km site the mean vector is oriented very accurately toward home (mean HDC = 0.86) but the scatter of the bearings is greater (mean LMV = 0.67) ; *i.e.,* the similar values for the homeward component at the two sites result from quite different behavior by the pigeons (Fig. 4). Similarly, a sharp drop in the value of the homeward component is seen at both the 143 km north site (Fig. 1A) and the 120 km west site (Fig. 1D), but the behavior of the pigeons at the two sites is fundamentally different. At 120 km west (Fig. 5A), the birds vanish randomly or nearly randomly (mean LMV = 0.44) but sometimes with a slight tendency to go in the opposite direction from home (mean HDC = −0.44), whereas at 143 km north (Fig. 5B), the birds always vanish nonrandomly and consistently show very little

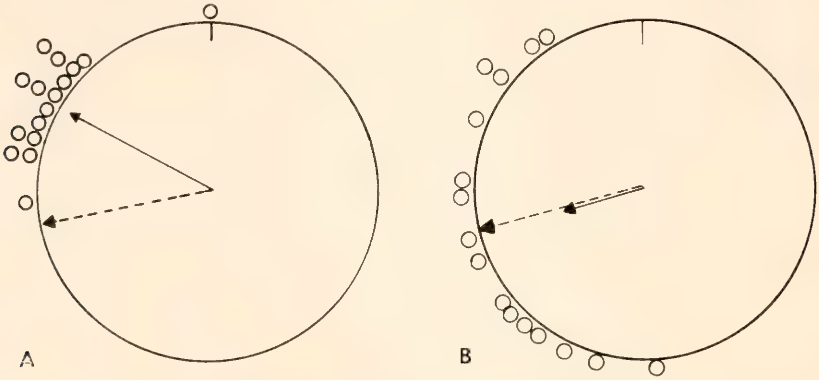

FIGURE 4. Release (A) showing very little scatter of the vanishing bearings but a mean deviating appreciably from the home direction, contrasted with a release (B) with much more scattered bearings but a more homeward directed mean; A, Berry Hill Fire Tower, New York, 37.9 miles from the loft, October 14, 1968, home bearing 258°, mean bearing 299° (nonrandom, $P < 0.0001$) ; B, near New Berlin, New York, 56.6 miles, July 30, 1967, home bearing 257°, mean bearing 256° (nonrandom, $P < 0.0001$).

scatter in their bearings (mean LMV = 0.81) but the mean vector is always oriented considerably west of the true home direction (mean HDC = 0.39).

Some re-analysis of Schmidt-Koenig's data in terms of homeward directedness of the mean vector and scatter of the bearings is possible. Thus, for example, if we look at the original data of Schmidt-Koenig (1966, page 41) from which Wallraff (1967, page 340) graphed the homeward component of Schmidt-Koenig's releases from the west, we discover that the nearly identical values for the homeward component shown for the releases at 30 km and 40 km in Wallraff's curve result from very different behavior on the part of the pigeons. The homeward component of 0.06 for 30 km is the average of the values (+0.64 and −0.51) for two releases both of which had bearings that were distributed nonrandomly ($P \leq 0.01$) but in which the mean vectors were oriented in nearly opposite directions, one homeward and one away from home. By contrast, the homeward component of 0.04 for 40 km is the average of the values (+0.23 and −0.14) for two releases both of which yielded random bearings. Thus emphasis on the homeward component has resulted in lumping together as similar examples of poor homeward orientation at intermediate distances values resulting from fundamentally different behavior. (Values for distances and for homeward components mentioned here are estimated from Schmidt-Koenig's (1966) figure and are thus only approximate.)

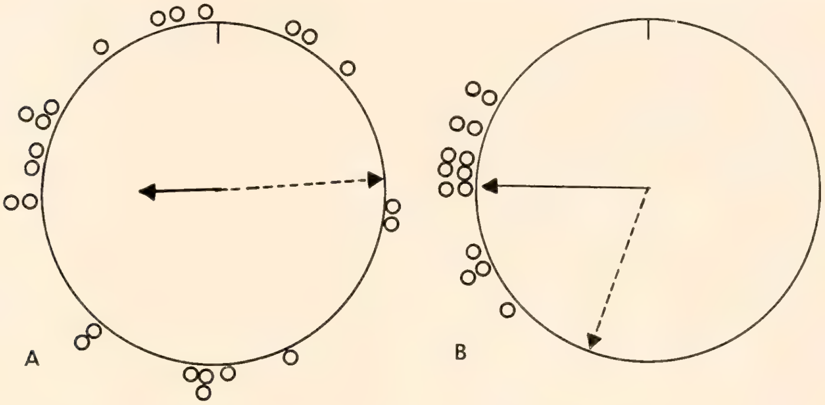

FIGURE 5. Release (A) showing very great scatter of the vanishing bearings, contrasted with a release (B) with little scatter but a mean deviating by a large angle from the home direction; A, Jersey Hill Fire Tower, New York, 74.5 miles from the loft, August 2, 1968, home bearing 85°, mean bearing 269° (random, $P = 0.287$); B, Castor Hill Fire Tower, New York, 89.1 miles, July 31, 1969, home bearing 200°, mean bearing 272° (nonrandom, $P = 0.0005$).

Wallraff's (1967, page 341) graph of distance effect in his own first-flight pigeons permits re-analysis in terms of homeward directedness of the mean vector and scatter of bearings. The results indicate that his data do not support the idea that *orientation* improves with distance. Thus his graph of releases from the east shows a rising homeward component at distances beyond 20 km, but this "improvement" turns out to be due largely to increased scatter in the bearings rather than to a more homeward-directed mean vector. In all but the first of his east releases, the mean vector is oriented away from home, hence the increasing scatter of his birds' bearings with distance results in a higher real value (but lower absolute value) for the homeward component. In short, there seems to be a trend toward randomness rather than toward improved homeward orientation.

The values for the homeward component obtained at one release site may differ significantly from those obtained at another site located approximately the same distance and direction from the home loft. For example, the mean homeward component at our 14.9 km east site (Fig. 1B) is 0.79, indicating very good homeward orientation. After conducting a series of five releases at this site (where the home direction is 247°), we performed a series of five releases at an alternate site 15.1 km east of the loft (home direction, 276°), and obtained a mean homeward component of 0.37, indicating much poorer orientation. Clearly the shape of the homeward component curve shown in Figure 2B would be quite different if the results at this alternate site were substituted for those at our usual site. The homeward component appears to depend upon peculiarities of the individual release sites that are not correlated with distance from the loft. Depending upon ones selection of sites, curves of many different shapes could be obtained when the homeward component is graphed as a function of distance.

It follows from this type of analysis that the homeward component is not a very useful basis for comparing pigeons' orientational behavior at different release sites. Much more meaningful information is provided by the combination of the homeward directional component and the length of the mean vector. However, our data indicate that neither of these varies consistently for our birds with the distance of the release site from the loft. Graue (1970) has recently also failed to find a distance effect in the orientation of his birds in Ohio. The distance effect does not appear to be a general phenomenon, and hence its theoretical implications are doubtful.

I wish to express my thanks to all of the following people who helped conduct the releases reported in this paper: Andre Gobert, A. Irene Brown, Lorraine Pakkala, Donald Windsor, Judith R. Alexander and Howard French. This research was financed by grant No. GB-13046X from the National Science Foundation.

SUMMARY

1. Matthews (1955, 1963), Schmidt-Koenig (1964, 1966, 1968), and Wallraff (1967) have reported that pigeons orient toward home best when released close to the loft or at a long distance from it; they report poor orientation at intermediate distances, and suggest that this has important implications for the nature of the navigational system used by birds.

2. We have failed to detect any such distance effect in 172 test releases utilizing 2525 single-tossed pigeons from the Cornell lofts. When the homeward component is plotted against distance, we obtain dissimilar curves for the four cardinal directions. Furthermore, the values of the homeward component may be quite different at two release sites approximately the same distance and direction from the loft.

3. We obtained particularly good orientation at the intermediate distances where, according to the distance effect, it should be poorest. This was true not only of experienced birds but also of first-flight youngsters.

4. Analysis of our data, as well as re-analysis of some of the published data of others, indicates that a much clearer picture of the behavior of the birds is given by the combination of the homeward directional component and the length of the mean vector. This permits segregation of the two factors that together determine the homeward component. These two measures vary independently, and neither shows the distance effect.

5. I conclude that the distance effect is not a general phenomenon.

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Note Added in Proof

While this paper was in press, Wallraff (1970, *Z. Tierpsychol.,* **27**: 303–351) published an analysis of his data in terms of the accuracy of the mean bearing and the length of the mean vector (see his page 340). Also, Schmidt-Koenig (1970, *Z. Vergl. Physiol.,* **68**: 39–48) has published further data from his studies of distance effect in Germany. Although he continues to report a distance effect in the homeward component, he does not find the same effect in either vanishing interval or, more importantly, homing speed. This suggests that his birds released at intermediate distances are correcting their bearings soon after being lost from sight, while they are still within the "zone of disorientation." If this is so, it contradicts the hypothesis that the birds must be either close to home or very distant from home to orient accurately.

Reference: *Biol. Bull.*, **139**: 520–538. (December, 1970)

CONTROL OF MOLTING IN MANDIBULATE AND CHELICERATE ARTHROPODS BY ECDYSONES

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Ecdysone and related steroids are the molting hormones of insects (*cf.* Novak, 1966; Kaplanis, Thompson, Robbins and Bryce, 1966; Kaplanis, Thompson, Yamamoto, Robbins and Louloudes, 1967; Thompson, Kaplanis, Robbins and Yamamoto, 1967; Williams, 1968). When 0.5 to 5 μg of these compounds are injected into isolated pupal abdomens of Cynthia moths, for example, they cause prompt development and the pupal abdomen molts into the corresponding fragment of an adult abdomen (Williams, 1968). The sequence of events triggered off by ecdysones in insects, which are collectively termed "molting," includes separation of the epidermis from the old cuticle (apolysis), the secretion of molting fluid, the secretion of a new cuticle, the digestion and resorption of part of the old cuticle, and the shedding of the old cuticle (ecdysis). Similar events occur in crustaceans during a normal molt cycle (Passano, 1960), but the control agents have not yet been identified. Evidence that ecdysones might be involved came from studies of Carlisle (1965) which showed that ecdysone extracts from different crustaceans and locusts caused molting in the crab *Carcinus maenas* from which Y-glands had been extirpated. Furthermore, ecdysones identical in their chemistry to those of insects were isolated from crustaceans (Hocks, Schulz and Karlson, 1967; Hampshire and Horn, 1966; Galbraith, Horn, Middleton and Hackney, 1968) and Lowe, Horn and Galbraith (1968) reported that injection of ecdysterone (= 20 hydroxy ecdysone or β-ecdysone) isolated from the crustacean, *Jasus lalandi*, caused shortening of the interecdysial period in eye-stalkless crayfish, *Procambarus simulans*. However, when we began these experiments, no reports existed of the successful induction of molting in arthropods other than insects by purified ecdysones.

The first example of ecdysone-induced molting in arthropods other than insects was the induction of molting in an isopod, *Armadillidium vulgare*, by ecdysterone (Krishnakumaran and Schneiderman, 1968, 1969). About nine days after receiving 150 μg/g of ecdysterone, the epidermis of the posterior parts of those isopods underwent apolysis and secreted a new cuticle and, two to three days later, underwent normal ecdysis. This was followed by secretion of a new cuticle and ecdysis of the anterior part, after which the animals resumed normal activity. The fact that both insects and crustaceans used ecdysones suggested that all mandibulate arthropods might use the same molecule for the control of molting. It was unclear, however, whether the other major subdivision of arthropods, the chelicerates, also use the ecdysones to control molting. The data in this report demonstrates that ecdysterone and several other ecdysones induce molting in crustaceans. These include cyasterone, ponasterone A, α ecdysone, inokosterone and an ecdysone analogue, β SEA 1. The data also show that diverse chelicerate arthropods including spiders, tarantulas and horseshoe crabs can be caused to molt by the injection of ecdysterone. The results also demonstrate that injected ecdysones cause a similar pattern of cuticle formation and molting in crustaceans, insects and arachnids. The possible evolutionary significance of a common path for the chemical control of molting in arthropods is discussed.

A preliminary account of these results was published earlier (Krishnakumaran and Schneiderman, 1968). In addition, Wright (1969) has recently demonstrated that ecdysone causes molting in ticks.

Materials and Methods

Experimental animals

Two species of crayfish of the genus *Procambarus* were used. One was collected locally in northeastern Ohio and the other was purchased from Schettle Biologicals, Stillwater, Minnesota. The Ohio forms weighed between 3 to 12 grams while Minnesota forms weighed between 10 to 22 grams. The crayfish were maintained in the laboratory in shallow aquaria containing tapwater. The temperature and photoperiod were those of the laboratory (24–26° C and 12–15 hrs of light). Elodea served as food and also helped in oxygenation. In addition, the animals were fed meat once every two weeks. They survived for long periods under these conditions and several of them molted spontaneously in early summer and late fall. These ecdysed normally and survived the molt, provided that they were isolated from their cannibalistic neighbors when they were newly ecdysed. The crayfish were maintained in the laboratory for several days before they were used for the experiments. This helped in determining the normal rate of molting for that season of the year. In certain seasons (*e.g.*, spring), it was necessary to wait until the crayfish molted once in the laboratory before they could be used.

Another crustacean used in this series of experiments was the fiddler crab, *Uca pugilator*. Specimens of *U. pugilator* were collected off Woods Hole and shipped to Cleveland in August where they were maintained in sand troughs containing "instant ocean," an artificial seawater (Aquarium Systems, Inc., Cleveland, Ohio). They were fed periodically on crab meat and survived for several months. Crabs in the process of molting could be recognized by their pale carapace and lethargic movements, and were isolated individually in small transparent plastic containers containing sand and instant ocean. If such crabs failed to ecdyse or died within a week after isolation, they were peeled and examined to determine whether or not they had secreted a new cuticle.

Three different chelicerate arthropods were also used. The horseshoe crab, *Limulus polyphemus*, the spider, *Araneus cornutus*, and the tarantula, *Dugesiella hentzi*. Specimens of *L. polyphemus* were collected off Woods Hole, Massachusetts in July and were maintained either in running sea water tanks at the Marine Biological Laboratory there, or in an aquarium containing "instant ocean." Animals weighing 30 to 50 g each or 3 to 12 g each were used. No food was given during the experiment except for debris that might have been brought in by the running sea water and from the seaweed and colonial invertebrates present in the aquarium. Animals were maintained at 19° C and received approximately 12 to 15 hrs of daylight. No effort was made to regulate photoperiod. Such animals survived several months under these conditions and several were still alive after a year in the artificial sea water.

The spider *Araneus* and the tarantula *Dugesiella* were obtained from Carolina Biological Supply, Inc. *Araneus* were collected in Oregon and were maintained individually in 30 ml plastic vials with screw caps. They weighed between 100 to 400 mg each and were sexually mature as evidenced by the production of egg cocoons. *Tenebrio* pupae were given as food weekly. The animals were kept in the dark at 18° C. These animals displayed no external morphological signs of molting prior to ecdysis. However, most of the spiders that ultimately secreted a new cuticle, stopped feeding and spun a molting pad which consisted of a few threads attached to the wall of the vial. Normally, these animals spin an orb web.

Tarantulas were maintained individually in glass tanks, the floor of which was covered with gravel. A cardboard tube served as a hiding place. They weighed between 8 to 12 g and both sexes were used. Live crickets and *Tenebrio* pupae were given as food weekly.

Hormones and other reagents

Ecdysterone and inokosterone were obtained from Rohto Pharmaceuticals, Osaka, Japan or Mann Research Laboratories, New York. α ecdysone was provided by Dr. P. Hocks, Schering, A. G. Berlin, ponasterone A was provided by Dr. John Pollard of Calbiochem. Cyasterone was provided by Professor C. M. Williams and came from the laboratory of Professor T. Takemoto, Tohoku University, Sendai, Japan. Drs. W. Robbins and Malcolm Thompson of the USDA at Beltsville, Maryland, provided the four ecdysone analogues: β SEA-1 (Δ^7-5β-cholestene-2β, 3β, 14α-triol-6-one), β SEA-4 (Δ^7-5β-cholestene-2β, 3β-diol-6-one), β SEA-12 (Δ^7-5β-sitostene-2β, 3β, 14α-triol-6-one) and α SEA-1 (Δ^7-5α-cholestene-2β, 3β, 14α-triol-6-one). Cholesterol and beta sitosterola were purchased from Nutritional Biochemicals, Cleveland, Ohio. Tritiated thymidine (Schwartz Bioresearch) was employed at a concentration of 1 mc/ml with a specific activity of 1.9 c/m Mole.

Ecdysterone and inokosterone were dissolved in insect Ringer (Ephrussi and Beadle, 1936), 10% ethanol or crustacean Ringer (Pantin's Ringer according to Marine Biological Laboratory Formulary, M.B.L., Woods Hole, Massachusetts) as the case may be. Cyasterone and ponasterone were dissolved in 10% ethanol, and α ecdysone in 20% ethanol. The ecdysone analogues were suspended in 20% ethanol, whereas the cholesterol and beta sitosterol were either dissolved in absolute ethanol or suspended in 50% ethanol.

Experimental procedures

Animals were anaesthetized in crushed ice and/or carbon dioxide. The materials were injected via a glass needle or 31 gauge steel needle using a Hamilton microliter syringe or a microburette. The volume of the material injected was usually less than 2 to 4% of the weight of the animal. Animals were kept for 2 to 4 hours at 5° C after injection before they were returned to the temperature at which they were normally maintained. Tritiated thymidine was injected at a dose of 10 μc/g live weight of the animal. The isotope was administered at specific times after the injection of ecdysterone and was allowed to circulate for specific periods of time before the animals were killed. Animals to be killed were sliced in half and plunged immediately into Bouin's fluid and processed to make 5 to 6 μ thick paraffin sections. Autoradiographic methods were similar to those described earlier (see Krishnakumaran, Berry, Oberlander and Schneiderman, 1967).

Each experiment was repeated at least once, and in several cases two or three times. In all cases the results obtained were comparable and the results of typical experiments are shown in the tables.

RESULTS

1. *Effects of ecdysterone on* Procambarus

In the first series of experiments, ecdysterone dissolved in Ringer solution was injected to a final concentration of 20 μg/g live weight. The data in Table I reveal that all the experimental animals molted within 10 days of the injection. The molt was abnormal, and in no case was there successful ecdysis and survival of the experimental animals. However, the animals did undergo apolysis and secreted a new cuticle which became obvious when the old cuticle was peeled away. All of the animals showed a swelling between the cephalothoracic shield and the abdomen. In a few cases the cephalothoracic shield separated from the new cuticle near its junction with the abdominal tergites.

TABLE I

Effect of ecdysterone on molting in the crayfish, Procambarus *sp.*

Chemical and dose	Number of animals	Per cent that molted within a month
Uninjected control	10	10
Ethanol 10% 4 μl/g	10	0
Cholesterol 20 μg/g	10	0
Ecdysterone 20 μg/g	10	100*
10 μg/g	10	100*
6 μg/g	10	100*
3 μg/g	10	100†

Animals weighed between 12 and 20 grams each.
* Molted within 10 days after injection.
† Molted within 14 days after injection.

In insects it is known that high doses of ecdysones are pathological and frequently cause the death of the injected animals (Kobayashi, Takemoto, Ogawa and Nishimoto, 1967; Williams, 1968). Presuming that the mortality observed in the preceding experiment may have been the result of a pathologically high dose of ecdysterone, lower concentrations of ecdysterone were injected. In one series of experiments we injected 3, 6, or 10 μg/g ecdysterone. Table I shows that even 3 μg/g of ecdysterone induced molting in *Procambarus*. However, even after such a low dose, almost all of the experimental animals died during ecdysis. Only two of the crayfish which received 3 μg/g ecdysterone completed ecdysis and survived. Even these were able to shed spontaneously only the caphalothoracic shield, and the remaining cuticle had to be peeled away.

Although between 3 and 20 μg/g of ecdysterone were effective in inducing molting processes, there were distinct differences in the response to low and high doses. Thus an injection of 6, 10, or 20 μg/g of ecdysterone resulted in apolysis and secretion of a new cuticle within ten days, whereas animals injected with 3 μg/g took about fourteen days to secrete a new cuticle. Crayfish receiving lower doses of ecdysterone also differed in the size of the gastroliths formed and the extent of digestion of the old cuticle. Gastroliths were best developed and the digestion of old cuticle most pronounced in crayfish that received 3 μg/g ecdysterone. The gross structure and histochemistry of the cuticles deposited in response to different doses of ecdysterone were identical, but certain morphological features of the cuticle were affected by the dose. Thus, the newly-deposited cuticle was similar to normal cuticle and contained a non-chitinous epicuticle, a chitinous, lamellated endocuticle and polyphenolase. However, animals that received 20 μg/g ecdysterone secreted a much thinner new cuticle than those that received lower doses of the hormone. Also, the bristles and hairs on the uropods and the branchial gills were shorter and ill-formed in all experimental animals except those that received only 3 μg/g ecdysterone.

An additional effect of high doses of ecdysterone was its inhibition of regeneration of appendages. Crayfish that had lost some of their appendages (usually walking legs) several weeks before hormone treatment, were injected with 20 μg/g ecdysterone. These animals molted promptly, between 7 and 10 days after they received the hormone, without regenerating the lost appendages. However, similar animals that received 2 to 3 μg/g ecdysterone formed a small regenerate. In contrast, crayfish whose appendages were amputed only 7 to 10 days prior to the injection of ecdysterone failed to regenerate even after treatment with 2 to 3 μg/g ecdysterone. These results are reminiscent of the situation in the wax moth *Galleria* (Madhavan and Schneiderman, 1969) where regeneration of imaginal wing discs in the last larval instar is promoted by low doses of ecdysone, but fails to occur after injecting high doses. In the crayfish, as in *Galleria*, ecdysone may be necessary for regeneration, but when applied in high doses it provokes molting so promptly that there is insufficient time for the cell divisions necessary for regeneration.

2. *Histological and autoradiographic studies of the crayfish epidermis under the influence of ecdysterone*

To determine when the various events of the molt cycle occurred in the epidermis of the crayfish under the influence of ecdysterone, *Procambarus* was injected with 2 μg/g ecdysterone, and three animals were killed and examined at 4, 24, 48, 96, and 144 hours after the injection of the hormone. Animals were processed for histological study and 4 to 5 micron thick paraffin sections were prepared and stained with Mallory's triple stain or Meyer's haemalum and eosin. A study of these sections revealed that the old cuticle had apolysed from the epidermis about 48 hrs after the injection of hormone. By 96 hrs after injecting the hormone, a new cuticle, approximately 8 to 10 μ thick in the tergite region, had already been secreted.

At this time the nuclei, which began to enlarge at the time of apolysis, were greatly enlarged. Associated with this nuclear enlargement was an increase in the size and basophilia of the epidermal cells. Events which occur after 96 hrs are difficult to analyze in the present experiments because they may have been associated with the pathological changes connected with the imminent death of the animals. Six days after the injection of ecdysterone, the epidermis in some of the crayfish showed further changes, such as a decrease in cytoplasmic volume. They resembled to some extent intermolt epidermis, except that in the normal intermolt animal the nuclei of epidermal cells are compact and lack chromatin granules.

Analysis of DNA synthesis in epidermis was undertaken next. For this purpose crayfish were injected with ecdysterone at a dose of 2 μg/g and immediately thereafter, or 4, 24, 48, or 96 hrs later, tritiated thymidine (10 μc/g) was injected. Individuals were killed both 2 and 24 hrs after the injection of isotope. None of the epidermal cells incorporated tritiated thymidine into their nuclei, although blood cells engaged in extensive DNA synthesis during this period, and many well-labelled blood cells were seen. Apparently, the ecdysterone at the levels used in these experiments does not induce DNA synthesis in the epidermis of these crayfish, although it induces molting. It is noteworthy also that muscles, nerve cells, and connective tissue failed to synthesize DNA.

TABLE II

Effect of ecdysone analogues and steroids on molting in the crayfish, Procambarus *sp.*

Chemical and dose	Number of animals	Per cent that molted
10% ethanol 3 μl/g	10	0
Ecdysterone 6 μg/g	10	100
Inokosterone 6 μg/g	6	100
Cholesterol 6 μg/g	16	0
Cholesterol 20 μg/g	15	0
β SEA-1 50 μg/g	6	50
α SEA-1 50 μg/g	6	0
β SEA-4 50 μg/g	6	0
β SEA-12 50 μg/g	6	0
β Sitosterol 20 μg/g	6	0

Animals weighed between 12 and 20 grams each.

3. *The specificity of ecdysterone in inducing molting*

Does ecdysterone induce molting in these crayfish because it is either the normal molting hormone or an analogue of the normal hormone, or, is it possible that its effects represent a nonspecific pharmacological action of steroids? To test this possibility, we injected crayfish with various sterols, such as cholesterol and beta sitosterol or ecdysone analogues such as β SEA-1, α SEA-1, β SEA-4, and β SEA-12. These agents have very little or no ecdysone effects in insects. The results are recorded in Table II. Crayfish injected with 6 or 20 μg/g cholesterol or 20 μg/g sitosterol or 50 μg/g α SEA-1, β SEA-4, or β SEA-12 did not molt. These data suggest that the effects of ecdysterone are not due to a nonspecific pharmacological effect of steroids. Even chemicals closely related to the ecdysones, such as α SEA-1, β SEA-4 and β SEA-12, do not induce molting (see Table II).

The only chemical that showed any molt-inducing effect in *Procambarus* was β SEA-1, which is structurally similar to the ecdysones except for the absence of hydroxyl groups on the side chain. This substance has ecdysone-like effects on the house fly and *Calliphora* (Robbins, personal communication). In *Procambarus* it caused molting in 50% of the crayfish into which it was injected. These animals developed fully-formed gastroliths, more-or-less completely digested their old cuticle and underwent spontaneous partial ecdysis, after which they died. Unlike ecdysterone, which induced prompt molting, β SEA-1 caused animals to molt 3 to 4 weeks after they received an injection.

4. *Effects of phytoecdysones on* Procambarus

From the preceding experiments it appears that ecdysterone induces molting in crayfish by specific hormonal action rather than by some nonspecific pharmacological effect. Is this activity limited to ecdysterone which normally occurs in crustaceans, or do the other zooecdysones and phytoecdysones that cause molting in insects also induce molting in crayfish? To test this, α ecdysone, inokosterone, ponasterone A and cyasterone were injected into *Procambarus*. An injection of 3 μg/g of any of these ecdysones induced molting in these crayfish. But as Table III shows, there were differences in the percentage of animals that responded and the time required for a response. Cyasterone was the least effective and induced molting in only 30% of the animals. In contrast, ecdysterone was the most effective and induced molting in all of the experimental animals. Inokosterone, α ecdysone and ponasterone were intermediate in effectiveness with 86%, 66% and 46% of the animals molting, respectively. When higher doses of cyasterone (6 and 10 μg/g) were injected, all of the treated crayfish molted, indicating that the response was dose-dependent.

TABLE III

Effects of different ecdysones on molting in the crayfish, Procambarus *sp.*

Treatment	Number of animals	Per cent that molted during the period indicated (approximate)			Cumulative % of crayfish that molted during the 21 day period
		7–10 days	10–14 days	14–21 days	
Uninjected controls	25	0	0	0	0
Controls injected with 10% ethanol 3 μl/g	10	0	0	0	0
Ecdysterone 3 μg/g	7	14	86	0	100
α Ecdysone 3 μg/g	15	56	7	7	66
Inokosterone 3 μg/g	14	21	56	7	86
Ponasterone A 3 μg/g	15	14	35	0	46
Cyasterone* 3 μg/g	10	10	10	10	30

Animals weighed 3 to 20 grams.

Most of the animals died in the process of molting.

* Higher doses, such as 6 μg/g and 10 μg/g, induced molting in 100% of experimental animals.

Another distinguishing feature of the different ecdysones was the interval between the time of injection of hormone and the induction of the molt. An injection of 3 μg/g of α ecdysone caused 56% of the animals to molt between 7 and 10 days, whereas in the case of ponasterone, inokosterone and ecdysterone, most of the animals molted between 10 and 14 days after injection. The reasons for this difference are not obvious; it is possible that α ecdysone is the true hormone with a short half-life, while other ecdysones may be less effective, but more stable, degradation products or analogues of α ecdysone (*cf.* King and Siddall, 1969).

Almost all of the crayfish that had been induced to molt experimentally, died after secreting a new cuticle. In an effort to increase their survival, we removed their eye stalks. The rationale behind this maneuver is the fact that eye stalks are known to produce and store a molt-inhibiting agent (*cf.* review by Passano, 1960). The site of action of this agent is not known, but if it acted upon epidermal cells, then its removal might permit better survival after ecdysis. Eye stalks were removed the day prior to, or immediately after, the injection of 3 μg/g of ecdysterone. However, survival after the molt was not increased.

5. *Effects of ecdysterone on* Uca pugilator

Another experiment tested the effects of ecdysterone on a marine crustacean, *Uca pugilator,* a semi-terrestrial fiddler crab. Fiddler crabs received 20 μg/g of ecdysterone dissolved in 4 μl of Ringer solution. Controls received 4 μl of Ringer solution or no injection. The results, recorded in Table IV, show that 90% of the experimental animals molted within 30 days, whereas only 16% of the injected controls and 14% of the uninjected controls molted during this period. Unlike the controls which underwent normal ecdysis, the experimental animals never underwent spontaneous ecdysis, although they showed apolysis, deposition of a new cuticle, secretion of molting fluid and partial resorption of the old cuticle. The experimental animals that had deposited a new cuticle could easily be recognized by their pale color and lethargic movements. When such crabs were peeled, their new cuticle was revealed, but they immediately died. Even if left for 10 days after the first appearance of pale color, they failed to undergo spontaneous ecdysis. Another feature of the experimental molt was that a large percentage of the animals responded within the same short span of time; namely, 14 to 18 days after injection of ecdysterone.

TABLE IV

Effect of ecdysterone on the induction of molting in the fiddler crab, Uca pugilator

Treatment	Number of animals	Per cent that spontaneously ecdysed during the specific period (*) or revealed a new cuticle after peeling			Cumulative % of crabs that molted during the 30 day period
		0–10 days	10–20 days	20–30 days	
Uninjected controls	15	0	7*	7*	14
Controls injected with 4 μl/g Ringer	30	10*	3*	3*	16
Experimentals injected with 20 μg/g ecdysterone in 4 μl Ringer	30	0	83	7	90

The cuticle formed in response to the injection of ecdysterone was similar in general appearance to normal cuticle and possessed epicuticle, a lamellated endocuticle and polyphenolase. However, experimentally-induced cuticle differed from the normal cuticle in several respects. In general, the induced cuticle was thinner than the normal cuticle. This may be in part the result of premature death, but appeared to be associated more with the artificial induction of molting. Another conspicuous difference was in the nature of the tubercles on the lateral borders of the dactylus and claw and the anterior ventral margins of the carapace. The tubercles in the experimental animals were much smaller and ill-formed. However, their distribution and orientation was identical to the pattern found in the old cuticle. Similarly, the bristles and sensory hairs at the borders of the appendages and on the general cuticular surface were short and ill-formed. In normal animals, the cuticle on the two sides of the maxilliped differ in thickness: the external surface has a thick cuticle (20 to 25 μ), whereas the internal surface has a thin cuticle (4 to 6 μ). In experimentally-treated animals the cuticle on both sides of the maxilliped is of the same thickness (4 to 6 μ). In addition, in normal animals, the external surface of the maxilliped bears tubercles, while in the experimentally-treated animals, there were either no tubercles or small tubercles.

TABLE V

Effect of ecdysterone on molting in Araneus cornutus

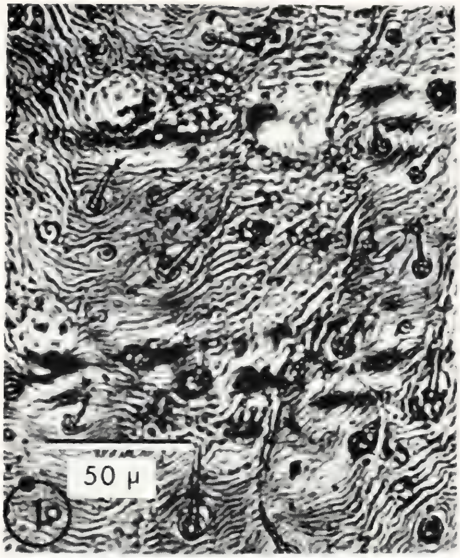
Treatment	Number of animals	Per cent that molted during the period indicated				Cumulative % of spiders that molted during the 29-day period
		0–8 days	8–15 days	15–22 days	22–29 days	
Controls injected with 4 μl of Ringer	24	8*	0	4*	0	13
Experimentals injected with 20 μg of Ecdysterone in 4 μl Ringer	29	7*	28	14	14	62

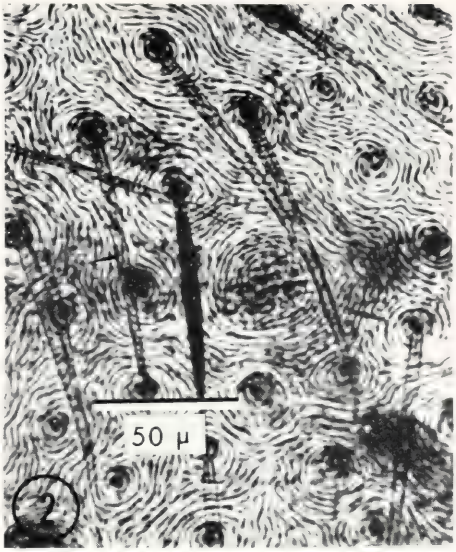
* Survived after spontaneous ecdysis. Others were peeled to determine whether they had molted.

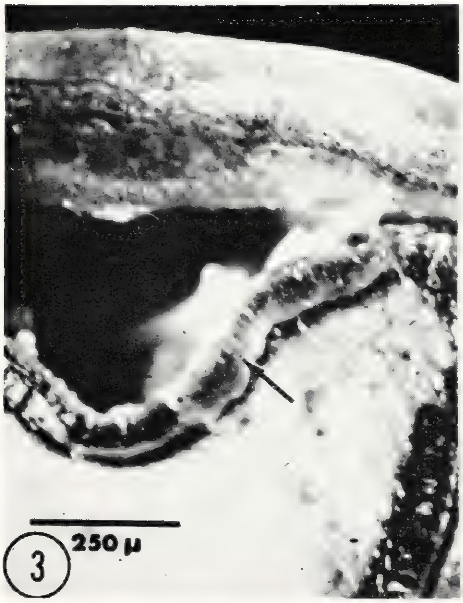
Control and experimental groups contained 29 and 30 spiders each, respectively. One of the experimentals and 5 of the controls died within the first three days after injection and are not included in the data. Sixteen controls and six experimentals survived for the duration of the experiment but did not show any signs of molting.

6. *Effects of ecdysterone on* Araneus cornutus

In the first series of experiments with the spider *Araneus,* animals were chilled on crushed ice and injected with 20 μg/animal ecdysterone in 4 μl of Ringers. The animals weighed approximately 150 mg, and thus the average dose of hormone was about 130 μg/g. Controls received 4 μl of Ringer solution. Results reported in Table V show that 62% of the experimentals molted in the 4-week period after injection, whereas only 13% of the controls molted. Unlike the controls which survived spontaneous ecdysis and continued normal life, the experimentals underwent apolysis and deposited a new cuticle, but then died. This was true of all the experimentally-treated animals that molted except for two that molted within







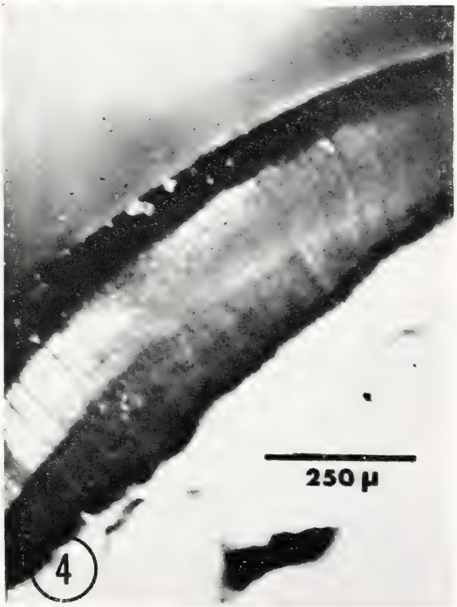


FIGURE 1. Surface view of cuticle from opisthosomal tergite in the spider, *Araneus cornutus* induced to molt by injection of ecdysterone. Note the short bristles and the inconspicuous ridges.

FIGURE 2. Same view as above from a control spider. The bristles are much longer and the ridges are conspicuous.

FIGURE 3. Cross section of telson from ecdysterone-injected *Limulus* showing the newly formed cuticle (arrow) inside the old cuticle.

FIGURE 4. Cross section of telson from control *Limulus* injected with Ringer.

the first eight days after receiving the injection of ecdysterone. Probably these had already begun spontaneous ecdysis before the hormone injection.

Frequently it was possible to recognize spiders that were about to secrete a new cuticle and molt by the fact that they ceased to feed and spun a molting pad rather than the normal orb web. Control spiders underwent spontaneous ecdysis, usually within a week after they ceased feeding and spun a molting pad, whereas the experimental spiders died. When the dead, hormone-treated spiders were peeled, it became evident that they had secreted a new cuticle. However, although the cuticle secreted after the injection of ecdysterone resembled normal cuticle in the arrangement of the basic cuticular layers and its staining properties, it was abnormal, particularly in its surface pattern and in the morphology of spines and bristles. While normal cuticle bears conspicuous ridges on its outer surface, in the experimental cuticles, the ridges were either shallow or absent. Spines which are long in normal cuticles were much reduced in length in the experimentals and frequently did not rise above the surface of the cuticle. However, the sockets bearing these spines appeared to be normal in number and distribution in experimental animals (Figs. 1 and 2).

In a second series of experiments, ecdysterone was dissolved in absolute methanol to a final concentration of 5 $\mu g/\mu l$ and applied topically to the surface of the abdomen. Each spider received either 4 μl of this methanolic solution or was dipped in a methanolic solution. The controls received either the same amount of absolute methanol or were dipped in methanol. Whereas 50% of the ecdysterone-treated spiders molted in a three week period, only 15% of the controls molted during this period. However, the mortality was much higher than after injection of ecdysterone in Ringer solution.

7. *Effects of ecdysterone on* Dugesiella hentzi

The primitive tarantula spider, *Dugesiella*, was used in the following experiments. A group of four tarantulas (2 males and 2 females), weighing between 8 and 12 g each, were injected with 200 μg of ecdysterone in 40 μl of Ringer solution, an effective dose of 16 to 25 $\mu g/g$. Controls (2 males and 2 females) received 40 μl of Ringer solution. Both groups remained active and fed normally, but neither the experimentals nor the controls molted during the 60-day period they were observed. Two months after the initial injections, three of the experimental animals were injected with a larger dose of ecdysterone—50 $\mu g/g$—and the fourth was injected with 100 $\mu g/g$. Controls received corresponding amounts of Ringer solution. Seven days after receiving the second injection of ecdysterone, one of the experimental animals, which received 50 $\mu g/g$, died. Dissection revealed that the animal had undergone apolysis but there were no signs of a new cuticle. Twenty days after the second hormone injection, another of the tarantulas underwent spontaneous ecdysis and survived. It secreted a normal cuticle replete with spines and bristles. (This same animal underwent a normal, uninduced, spontaneous molt ten months later and is alive at the time of writing). Between 22 and 28 days after the second hormone injection, the other two hormone-treated animals also molted. These showed both apolysis and the secretion of a new cuticle, but did not undergo spontaneous ecdysis. Like the old cuticle, the new cuticle possessed a distinctive fuchsinophilic epicuticle and an aniline blue-stained lamellated endocuticle. However, it differed from the normal cuticle in the absence of bristles and spines. The controls survived for several months without molting. One of these was killed two months after the second injection of Ringers and there were no signs of initiation of a molt. Since the molts in all four experimental animals were induced in September, three months after the normal natural period of molting in these animals, and since none of the four controls molted spontaneously during this period, it seems clear that the injection of ecdysterone induced the molt.

8. *Attempts to demonstrate effects of ecdysterone on isolated abdomens of spiders*

One sure way to determine whether ecdysterone is responsible for the induction of molting in insects has been the testing of the hormones on isolated abdomens which are devoid of the major endocrine glands. The morphology of spiders appeared to make them particularly amenable to such a surgical maneuver. Although we do not know the source of the molting hormone in the spiders, we assumed that the glands responsible for molting are not located in the abdomen. With this gratuitous assumption in mind, we prepared a number of isolated abdomens of *Araneus* and *Dugesiella* and some other common spiders by ligating the narrow waist-like opisthosoma. The anterior halves of these animals were excised and, after applying penicillin, streptomycin and phenylthiourea, the wound was sealed with paraffin. These isolated abdomens survived for a week to ten days. However, injection of 20 μg of ecdysterone failed to cause molting. These unsuccessful results are reported only because this may be the first report of such surgical operations on spiders and may prove useful for some other study. The negative results we obtained may reflect the fact that we performed only a limited number of operations.

TABLE VI

Effects of ecdysterone on molting in Limulus polyphemus

Treatment	Number of animals	Per cent showing signs of molt within one month	Remarks
Uninjected controls	50	2	Only apolysis
Controls injected with 8 μl of Crustacean Ringer	6	0	—
Experimental injected with 40 μg/g ecdysterone in 8 μl Ringer	5	100	Secreted new cuticle but had to be peeled

Animals weighed between 35 and 50 grams each and were kept in running sea water.

9. *Effects of ecdysterone on* Limulus polyphemus

The horseshoe crab, *Limulus* is a relict merostomate arachnoid, only remotely related to modern arachnids, such as spiders and scorpions. Living genera of horseshoe crabs resemble closely the ancient genus *Paleolimulus* which lived some 200,000,000 years ago in the Permian. Their closest relatives are thought to be the extinct eurypterids and trilobites. Large specimens of *Limulus* which weighed 35 to 50 grams each, were injected with 40 μg/g ecdysterone dissolved in crustacean Ringer. Controls were either injected with a corresponding amount of Ringer or were uninjected. The results recorded in Table VI show that all experimentals molted. Only one of the fifty uninjected controls and none of six injected controls showed any signs of molting. Even the one control animal that showed signs of molting, underwent only partial apolysis. In *Limulus*, as in the other animals studied in this report, the experimentally-induced molt was different from a normal molt. The animals underwent apolysis and became paler in color but they never spontaneously shed their cuticles. Microscopic examination of such animals after peeling the old cuticle confirmed that the new cuticle had the same cuticular layers as the old cuticle (Figs. 3 and 4). Treatment of a second batch of smaller specimens of *Limulus* (3 to 10 grams) gave similar results.

These two series of experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts, where the specimens of *Limulus* were kept in tanks of running sea water. When the experiments were repeated in the laboratory at Cleveland, Ohio, using artificial sea water in a circulating system, they were unsuccessful. However, all 25 of the ecdysterone-injected specimens of *L. polyphemus* died within four weeks, whereas 60% of the 25 controls injected with Ringer survived for more than four months. None of the controls or the experimental animals deposited a new cuticle.

Discussion

1. *Ecdysones as the true molting hormones of all arthropods*

The data show that ecdysterone causes molting in diverse chelicerate and mandibulate arthropods. The effects appear to be true hormonal effects and not nonspecific effects of steroids. For example, less than 3 μg/g of ecdysterone caused molting in crayfish, whereas doses up to 50 μg/g of steroids such as cholesterol, beta sitosterola and the ecdysone analogues (β SEA-4, β SEA-12), which are hormonally inactive in house fly and *Calliphora* assays, have no effect in crayfish. A second reason for believing that the ecdysones are the true molting hormones of these arthropods is the occurrence of α ecdysone, deoxyecdysone, ecdysterone and at least two other ecdysones in crustacean (Galbraith *et al.*, 1968; King and Siddall, 1969; Faux, Horn, Middleton, Fales and Lowe, 1969). Although their presence in chelicerate arthropods has not been uncovered thus far, it seems likely that ecdysones occur in these arthropods as well. Thirdly, the dose required to induce molting in insects (Williams, 1968; Krishnakumaran, Granger and Schneiderman, 1970) and crustaceans (present results) is in the same physiological range.

A fourth line of evidence that supports the view that ecdysones are the natural molting hormones of arthropods other than insects is the abnormality of the cuticle deposited in response to ecdysone treatment. The experimentally-induced cuticles of spiders and crabs show several features which are similar to the pathological effects of high doses of ecdysones in insects. Such pathological effects in insects have been termed "hyperecdysonism" by Williams (1968) and appear to represent the first example of hyperhormonism in invertebrates. In Lepidoptera, these features include short, ill-formed bristles and scales, a decreased number of bristles and scales, an abnormal cuticular texture, and have been attributed to a telescoping of the normal sequence of synthetic events in which epidermal cells engage during the process of cuticle deposition. In the present experiments similar abnormalities were found in spiders (fewer and shorter hairs and bristles) and in fiddler crabs (reduced size of knobs on dactylus and on the anterior ventral surface of the carapace). These abnormalities appear to be caused by the abnormal dosage and timing of the application of the hormones: Normally ecdysone is released gradually, but in these experiments it was applied all at once. It seems that such "hyperecdysonic" effects would be expected only if ecdysterone were either a true natural molting hormone or of similar structure to the true molting hormone. The following facts also support this opinion.

If ecdysone caused some general pathological effect which resulted in abnormal cuticles, it might be effective even after the process of molting had begun. This is not the case. Injection of a high dose of ecydysterone after the initiation of molting has no "hyperecdysonic" effects in insects. The same appears to be true of the spiders which molted during the first eight days after injection of the ecdysterone. These spiders ecdysed spontaneously and produced a normal-looking cuticle, apparently because molting had been initiated prior to the injection. Thus, it appears reasonable to presume that the action of ecdysterone in the crayfish, and possibly in the other arthropods, is a true hormonal action.

What are the targets of ecdysone? In insects the targets of ecdysone include the epidermis (Wigglesworth, 1957) midgut (Piepho, Holz and Jung, 1964) nervous system (Pipa, 1969), imaginal discs (Madhavan and Schneiderman, 1969), several internal organs (Sehnal and Schneiderman, 1970) and sometimes the ecdysial glands (Schneiderman and Gilbert, 1964). The target tissues of ecdysones in other arthropods are probably the same, but certainly include the epidermis, for the following reasons. It is unlikely that the ecdysones cause molting in these diverse arthropods by activating the animal's own ecdysial glands, for if this were the case, one would not expect abnormal cuticles. When the ecdysial glands of insects are activated, by whatever means, normal cuticles are produced, provided one does not inject excess amounts of ecdysones. The abnormalities produced in the cuticle of these diverse arthropods by excessive amounts of ecdysone, suggest a direct action of the ecdysones on the epidermal cells. Apparently, at least the chitogenous epithelium of most arthropods is capable of responding to insect ecdysones. This implies that the final common path of the control of molting in most arthropods is the same, involving an ecdysone or ecdysone-like molecules and the associated receptor sites in the epidermal cells. Although definitive evidence for true homology must await the identification of the ecdysial glands in, and the isolation of ecdysones from chelicerate arthropods, the evidence presented in this report points to such homology.

The nature of the primary action of ecdysones on insect epidermal cells remains to be identified (*cf.* discussion in Krishnakumaran *et al.*, 1967). However, the results of the autoradiographic experiments on crustaceans reported here, which demonstrate that the epidermal cells of crayfish can secrete a new cuticle without first engaging in DNA replication, emphasize that the fundamental role of ecdysones as molting hormones may be uncoupled from any role they have have as growth hormones. Similar molts without DNA replication have been observed in adult insects (Krishnakumaran and Schneiderman, 1964; Krishnakumaran *et al.*, 1967) and in insects treated with high doses of ecdysones (Krishnakumaran, Granger and Schneiderman, 1970). It remains to be demonstrated when DNA replication normally occurs in the interecdysial period of crustaceans.

It is of interest that ecdysterone caused behavioral effects in addition to its effects on the cuticle. Spiders repsonded to ecdysterone by spinning a molting pad before any obvious changes in the cuticle were evident. Whether these are direct effects on the nervous system, analogous to those caused by sex hormones in vertebrates, or some indirect effects remains to be proven.

2. *Relative activities of different ecdysones*

In insects and in crayfish the active ecdysones were effective at doses varying between 0.5 to 5 $\mu g/g$ live weight. In chelicerate arthropods, higher doses appeared to be necessary. Injections of about 40 to 50 $\mu g/g$ were required to initiate molting in tarantulas and in *Limulus*. Perhaps the specific ecdysones employed by chelicerates are different from their counterparts in insects and crustaceans.

From a study of the relative activities of the five different ecdysones, it appears that ecdysterone is the most active in crustaceans, followed by inokosterone, α ecdysone and ponasterone A, with cyasterone being the least active. This contrasts with observations on some insects where cyasterone is among the most active of the ecdysones. For example, in the lepidopteran, *Samia cynthia,* the relative activities of the ecdysones are: cyasterone > ponasterone A > α ecdysone > ecdysterone > inokosterone (Williams, 1968). It is of interest also that β SEA-1, which is the least active of the active ecdysone analogues in insects, is also the least active ecdysone for crustaceans.

From the analysis of all the dose-effect data presently available to us, it appears that almost all of the ecdysones which are active in one group of arthropods will have some activity in other groups. Whether injected ecdysones actually affect target cells themselves, or are metabolically converted into other ecdysones which affect target cells, is unknown. Indeed, even in insects, the role of the interconversion of the several ecdysones is still unclear (*cf.*, for example, King and Siddall, 1969).

3. *Survival after experimental molt*

Insects which are caused to molt by injections of ecdysones commonly fail to survive. This is true also of most of the other arthropods examined here. In fact, only two each of the crayfish and spiders and one of the tarantulas which were caused to molt by ecdysones, survived for long periods after the molt. It is possible that the experimentally-induced molts caused death because of hyperecdysonism or because of the absence of certain necessary preparations for molting controlled by other hormones (such as the brain).

What are the probable causes of death in ecdysone-induced molts? In insects abnormal molting results in a defective cuticle in which wax layers may be incomplete and animals die because of desiccation (*cf.* review by Schneiderman, Krishnakumaran, Bryant and Sehnal, 1969). In other arthropods, defects in cuticle may have manifold effects. In crayfish, such defects may decrease their waterproofing and make them subject to dilution by fresh water. Both in spiders and in crustaceans, the presence of the old cuticle plus molting fluid over the book lungs or gills will decrease the effectiveness of these respiratory organs by increasing the distance across which gases must diffuse. Undoubtedly, there are other causes of death, but it appears likely that many are associated with cuticle. Ecdysones themselves do not appear to be toxic unless they cause molting, and animals which received injections of ecdysone rarely died except in the process of molting.

The only crayfish that survived were those that received a low dose of ecdysterone. Perhaps this activated their own ecdysial glands, or the dose was in the physiological range. The crayfish that died showed abnormal calcium resorption as indicated by poor formation of gastroliths and incomplete resorption of the old cuticle. These crayfish never shed their cuticles, possibly due to a failure in the absorption of water which normally precedes ecdysis.

These results are in marked contrast to those we obtained with terrestrial isopods in which normal molting was induced in more than half the animals by injecting ecdysterone (Krishnakumaran and Schneiderman, 1969). What are the reasons for the survival of these isopods and the death of other hormone-treated arthropods? One reason may be the peculiar way in which isopods molt. Posterior and anterior halves of these pill bugs undergo ecdysis consecutively, 3 to 4 days apart. This may involve either (a) a mechanism to remain insensitive to high levels of ecdysones circulating in the hemocoel, or (b) a controlling device that regulates the time of response of the chitogenous epithelium or (c) a mechanism to inactivate the excess ecdysone or (d) a combination of the above. Such a mechanism would prevent any hyperecdysonic effects and thus permit better survival. Another possibility is that the ecdysone activates the animals' own ecdysial glands.

4. *Phylogenetic considerations*

The fact that the ecdysones are capable of inducing molting, not only in insects but also in diverse crustaceans and chelicerates, suggests close similarity or even identity of the mechanisms that control molting in arthropods. If this proves to be true, it may throw some light on the phylogeny of the arthropods. The similarity in the chemistry of the molting hormone, and by implication the receptor sites in the chitogenous epithelium, strongly suggests the common ancestry of all arthropods. Manton (1964) and earlier Tiegs and Manton (1958) contend that the arthropods are polyphyletic in origin. They argue that the similarities in the structure and chemistry of the cuticle (see Richards, 1951; Krishnakumaran, 1961) independently evolved by convergence. The fact that the mechanisms controlling secretion of the cuticle are also homologous, makes such an argument unlikely. The convergence hypothesis becomes even less tenable when one adds the fact that the ecdysial glands of both insects and crustaceans have similar origins from the ectoderm of the embryonic cephalic region (see Jenkin, 1962; Herman, 1967). We have no knowledge of the location, structure, and origin of the ecdysial glands in the chelicerates. Should the ecdysial glands of chelicerates prove to be of epidermal origin and arise from the prosomic region, it would establish beyond all reasonable doubt that the arthropods are truly a homogeneous group with a monophyletic origin.

However, if it turns out that ecdysones are present and function in a wide variety of invertebrates such as annelids, nematodes, priapulids and related aberrant schizocoelic groups, then it is still possible that different arthropod groups may have evolved independently from several diverse post-annelid, prearthropod ancestors. It will be of interest to investigate the distribution and effects of ecdysones in annelids and other groups.

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SUMMARY

1. The ability of ecdysones to induce molting in arthropods other than insects was examined in representatives of both the mandibulate and chelicerate subphyla.

2. All five ecdysones tested caused molting in the fresh-water crayfish, *Procambarus.* Their relative activities were: ecdysterone > inokosterone > α ecdysone > ponasterone A > cyasterone. Doses as low as 3 μg/g of ecdysterone caused 100% of all test crayfish to undergo apolysis and secrete a new cuticle within 14 days after injection, but only in a few cases did the animals shed their old cuticles spontaneously after experimental treatment. At higher doses the new cuticle was thinner than normal and had abnormal bristles. The stimulation of molting was specific for ecdysones and was not copied by a variety of ecdysone analogues or other steroids.

3. Histological and autoradiographic studies revealed that ecdysterone at the levels used in these experiments caused molting in crayfish without DNA replication.

4. Ecdysterone also caused molting in the marine fiddler crab, *Uca pugilator.*

5. Ecdysterone caused molting in several chelicerate arthropods including the spider, *Araneus cornutus,* the tarantula, *Dugesiella hentzii* and in the horseshoe crab, *Limulus polyphemus,* which is among the most primitive of all living arthropods.

6. In spiders the ecdysone caused behavioral effects before any obvious changes in the cuticle were evident.

7. In almost all cases, molts induced by ecdysone were characterized by abnormal cuticles similar to those produced by injections of ecdysones in insects, a result which suggests a direct action of ecdysone on the epidermal cells. Most of the experimental animals failed to survive the molt and few underwent spontaneous ecdysis. These effects probably result from the abnormal delivery of a large amount of hormones in one dose, in contrast to the gradual release of hormone *in situ.*

8. From an analysis of all of the dose-effect data, it is concluded that almost all of the ecdysones which are active in one group of arthropods will have some activity in other groups. Since spiders and horseshoe crabs require doses about ten times as high as those needed for mandibulate arthropods, the specific ecdysones employed by the chelicerates may differ from their counterparts in insects and crustaceans.

9. The evidence suggests that ecdysones are the normal molting hormones of all arthropods and supports the view that arthropods have a common ancestry and are not a polyphyletic group.

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GASTRULATION IN THE TURBELLARIAN *HYDROLIMAX GRISEA* (PLATYHELMINTHES; PLAGIOSTOMIDAE): FORMATION OF THE EPIDERMAL CAVITY, INVERSION AND EPIBOLY[1]

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Gastrulation of the commensal turbellarian, *Paravortex gemellipara*, was defined by Ball (1916, p. 507) in the statement: "If by the process of gastrulation is meant the infolding or overgrowth of entoderm by ectoderm, then true gastrulation occurs. . . ." Ball's definition covers all processes, direct or indirect, by which ectoderm achieves its definitive position in the developing turbellarian. Entolecithal embryos of acoels and of polyclads gastrulate by epiboly or overgrowth by micromeres of yolk-bearing macromeres and their derivatives (Bresslau, 1933; Kato, 1940, 1968). Ectolecithal embryos of rhabdocoels, alloeocoels and triclads gastrulate in various ways, all of which may well represent adaptations to or modifications by the yolk cells which surround the developing embryo (Kato, 1968).

According to Ball (1916) the first step in the gastrulation of the ectolecithal embryo of *P. gemellipara* is the differentiation of the ectoderm. The outer cells of the embryonic mass become flattened and stretch posteriorly to entrap the endodermal cells which have enveloped the yolk cells. Ball concluded that gastrulation of *P. gemellipara* occurs by epiboly. Bresslau (1904) had earlier observed a similar process in *Mesostoma*. As cited by Hyman (1951, p. 175), Bresslau observed that the peripheral cells of the venter of the embryo of *Mesostoma* ". . . arrange into a surface epithelium, which is the ventral epidermis and which gradually spreads dorsally to enclose the dorsal yolk mass." The gastrulation of *Monocelis fusca* is accomplished by the differentiation and spreading of eight blastomeres (six *Hüllzellen,* two *Vitellocytophagen*), which form a peripheral embryonic epithelium (Giesa, 1966). The *Vitellocytophagen,* at the vegetal pole, engulf yolk cells, and are subsequently overgrown by four spreading *äquatorialen Hüllzellen.* The vegetal *Hüllzellen* and additional *Hilfzellen* transfer the remaining yolk cells into the body of the embryo. The definitive epidermis of *M. fusca* is formed when blastomeres move into the embryonic epithelium from the embryonic mass. The blastomeres differentiate into the epidermal cells, the nuclei of which sink back into the parenchyma (embryonic mass) of the turbellarian.

Gastrulation of triclad embryos involves the formation of transitory structures which do not contribute to the definitive organism (Bresslau, 1933; Kato, 1968). Blastomeres form a thin outer provisional ectoderm around the central yolk syncytium after the yolk cells aggregate. At one point on this provisional ectodermal sac a transitory pharynx forms by accumulation of blastomeres. A thin-walled cavity forms at the inner end of the temporary pharynx. The cavity is lined by a thin layer of cells, the internal membrane. The external cellular yolk cells are sucked through the transitory pharynx into the thin-walled cavity, now termed the temporary intestine. Between the wall of the temporary intestine and the provisional ectoderm, the definitive epidermis differentiates from proliferating blastomeres and spreads to replace the degenerating cells of the provisional outer and inner membranes.

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A remarkable process of gastrulation has been observed in a series of studies on the developmental cycle of *Hydrolimax grisea*. *Hydrolimax* is the only fresh-water member of the alloeocoel family Plagiostomidae reported from North America (Hyman, 1938). First described by Haldeman (1843), *Hydrolimax* was rediscovered by Hyman (1938) in collaboration with Ulric Dahlgren of

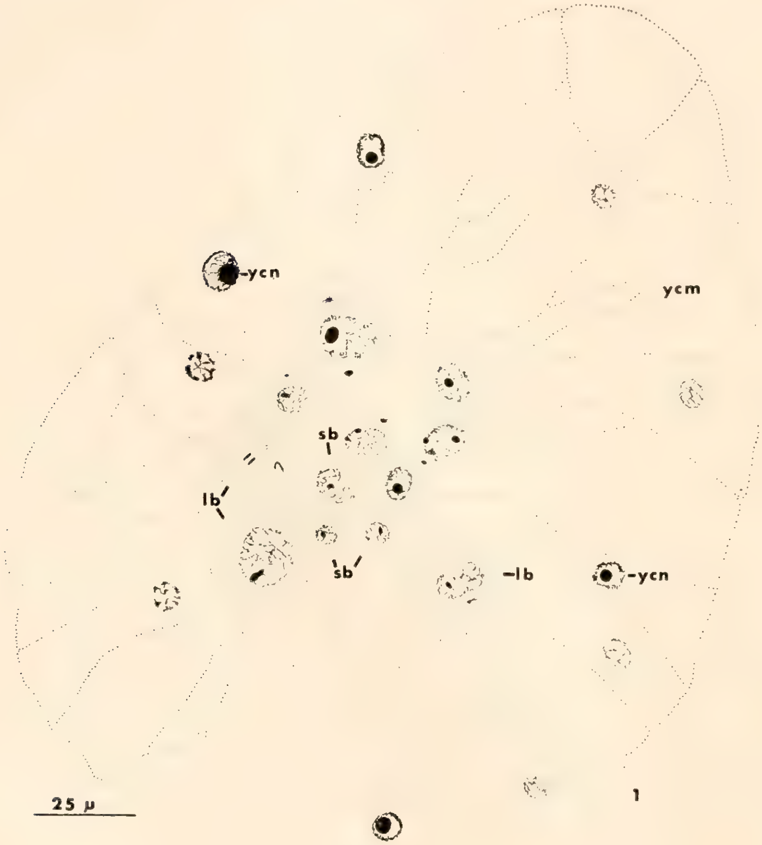

FIGURE 1. Cleaving embryo of *Hydrolimax grisea* enveloped by radially arranged (and closely adhering) yolk cells. Note the arrangement of the blastomeres: the smaller blastomeres are situated in the center of the embryo (embryonic mass); Costello's fixative, 4° C; lb, large blastomere; sb, small blastomere; ycm, yolk-cell mass; ycn, yolk-cell nucleus.

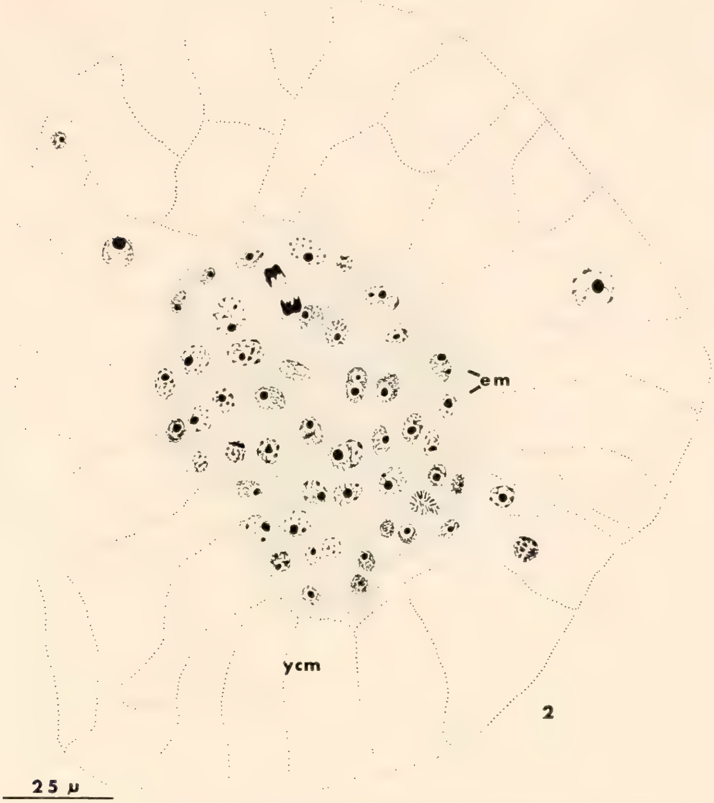

FIGURE 2. Older embryo: a solid mass of blastomeres situated in the center of the yolk-cell mass. Slightly older embryos are bilobed; Heath's polyclad fixative, 4° C; em, embryonic mass.

Princeton University. Hyman (1938) gave an account of the morphology and taxonomy and reviewed the scant literature of this turbellarian. A review of the more recent literature and a study of the oögenesis of *Hydrolimax* is presented in an earlier paper (Newton, 1970).

MATERIAL AND METHODS

The cocoons of *Hydrolimax* are characteristically red-brown in color and surprisingly large (up to 2 mm in diameter) for the size of the adult turbellarian (4 to 10 mm in length; 1 to 2 mm in width), as noted by Hyman (1938). They are deposited on hard surfaces, the underside of rocks, leaves, on bark of submerged tree trunks and rarely on emergent plants. The cocoons are deposited in the early spring, late March or mid-April, to June.

The material for this study was collected from two sources, Little Creek, east of Chapel Hill, and Morgan Creek, below University Lake impoundment west of Chapel Hill, North Carolina. Prior to fixation the cocoon capsules were

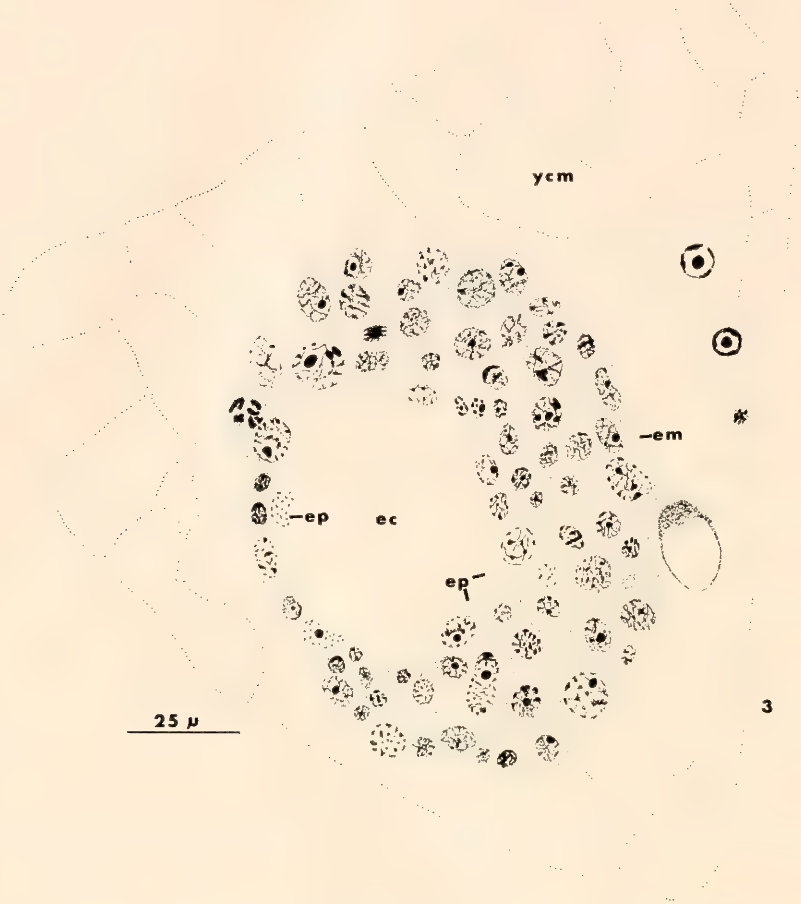

FIGURE 3. Embryo with expanding epidermal cavity lined by lightly-staining epidermal cells. The embryo is approaching the surface of the yolk-cell mass; Worcester's fixative, 25° C; ec, epidermal cavity; ep, epidermis.

punctured with insect pins to permit penetration of fixatives, as the capsule proved impermeable to the fixatives. During washing, the cocoons were opened up more to aid in washing, dehydration and infiltration. The specimens were placed in stender dishes and flooded with one of the following fixatives: Bouin's, Allen's, Worcester's, Heath's polyclad fixative and D. P. Costello's modification of Heath's fluid (sat. mercuric chloride, 81 parts; formalin, C. P., 9 parts; glacial acetic acid, 5 parts). The fixatives were used at initial temperatures of 25° C and 4° C. In addition some material was fixed in Bouin's at 37° C. The cocoons remained in the fixatives from 4 to 12 hours. Material fixed in Bouin's and Allen's fluids was washed in 70% ethanol until picric acid no longer leached out. Material fixed in Worcester's, Heath's and Costello's fluids was washed in several changes of distilled water for a period slightly longer than the time of fixation.

The cocoons were dehydrated, cleared, and infiltrated and embedded in filtered Paraplast (M. P. 56–57° C). Serial sections, at 8 μ, were cut. The sectioned material was stained with Heidenhain's iron-haematoxylin according to the procedures and recommendations of McClung and Conn (1937). Material fixed in Bouin's and Allen's fluids was more difficult to extract, taking longer, and, in the final stages of extraction, more difficult to control. Drawings were made with the aid of a camera lucida attached to a Spencer student microscope with a 4 mm objective and 15 × ocular.

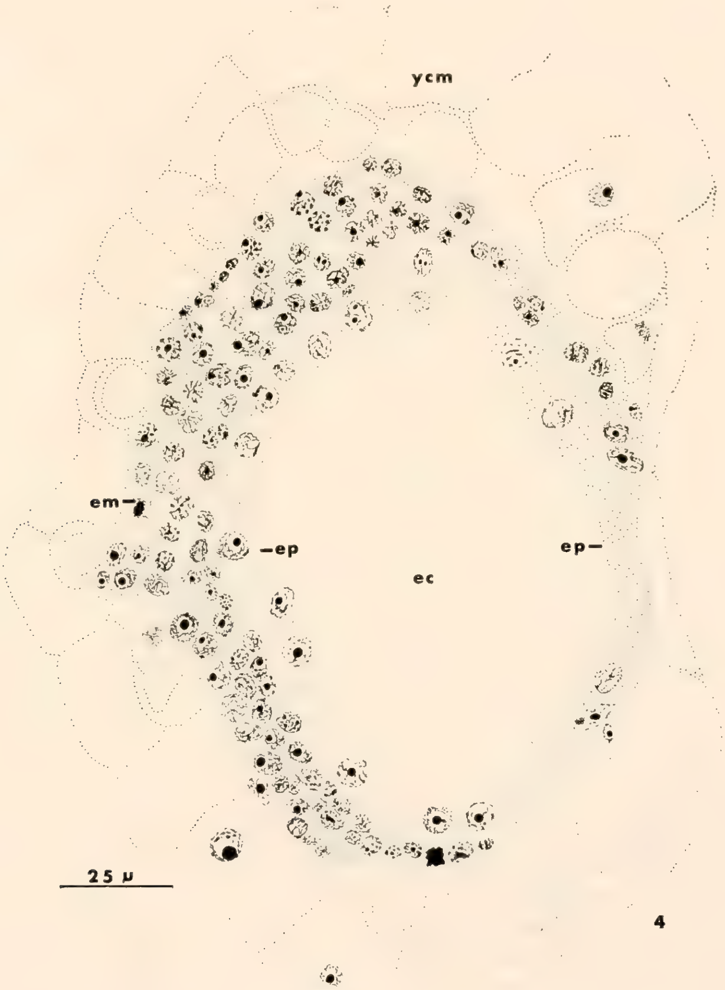

FIGURE 4. Epidermal cavity has expanded, forcing the overlying yolk cells aside. The epidermis nearest the surface of the yolk-cell mass will thin and become perforated. Note increased number of epidermal cells; Worcester's fixative, 25° C.

Some shattering of sections by cocoon capsule fragments did occur, but, in general, excellent serial sections were obtained.

OBSERVATIONS

Hydrolimax grisea, an hermaphroditic animal, practices mutual insemination by injection of spermatozoa through the wall of the genital atrium into the adjacent parenchyma (Hyman, 1938; Kepner, Stirewalt and Ferguson, 1941; Stirewalt, Ferguson and Kepner, 1942). The spermatozoa make their way through

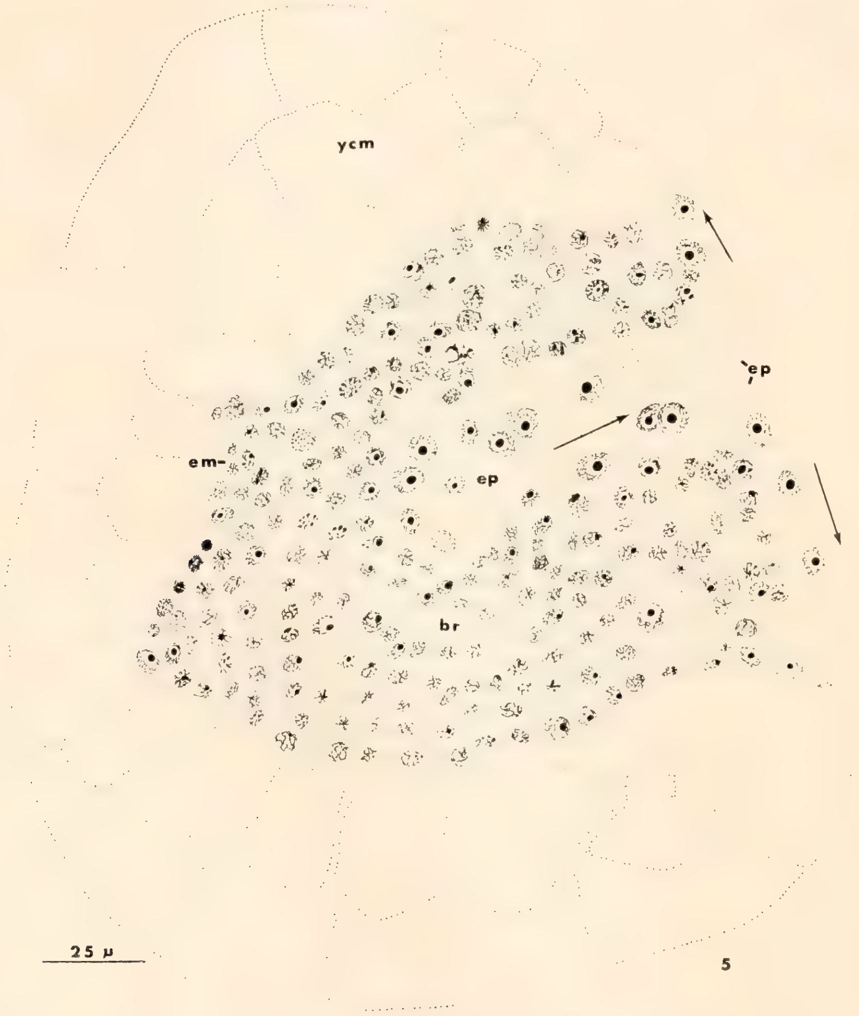

FIGURE 5. Epidermal cavity has collapsed and the embryo is in the process of inversion. The embryo rises out of and spreads over the yolk-cell mass (arrows); Worcester's fixative, 25° C; br, brain.

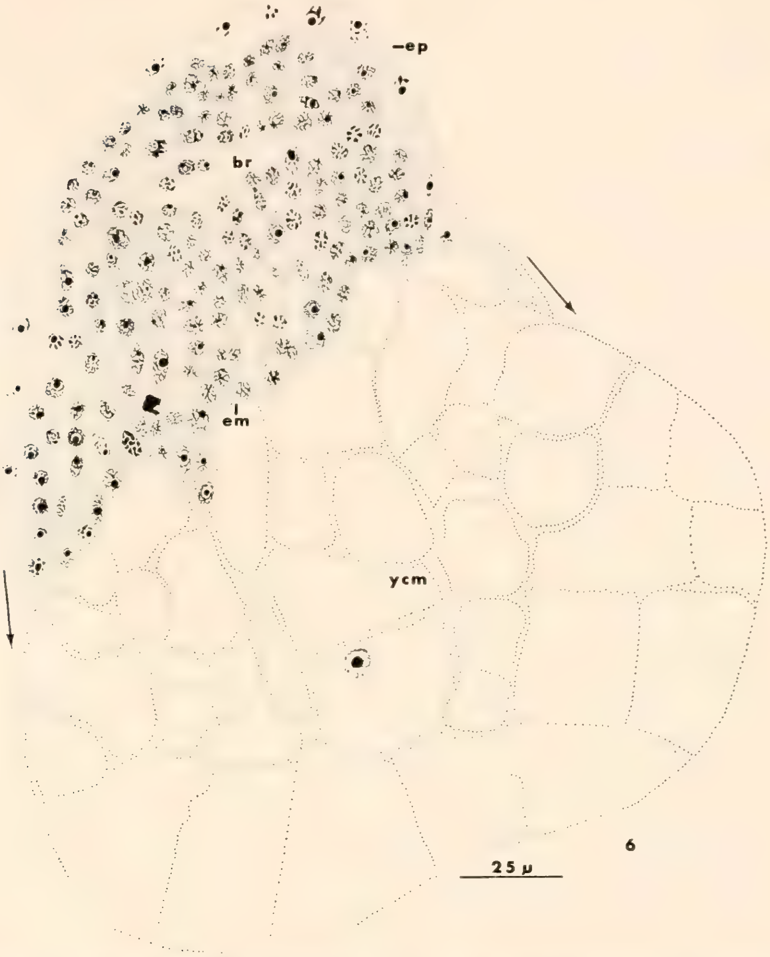

FIGURE 6. Inverted embryo of *Hydrolimax*. The lightly-staining epidermis is spreading posteriorly to entrap the yolk-cell mass; Bouin's fixative, 25° C.

the parenchyma of the adult turbellarian to the oöcytes (Hyman, 1938). At no time do the spermatozoa enter into the oviduct, as I have observed in studies of many sectioned specimens of mature *Hydrolimax*. As described earlier (Newton, 1970), the oöcytes are fertilized prior to breakdown of the germinal vesicle. The fertilized oöcytes migrate to the common genital atrium where 9 to 36 of them are incorporated, along with many yolk cells, into the cocoon. At the time of cocoon formation, the oöcytes are in meiotic prophase I (Newton, 1970).

Prior to the maturation divisions of the eggs, the yolk cells are spherical and loosely arranged around the oöcytes. By meiotic metaphase I, the yolk cells elongate and become radially arranged around the egg. They adhere closely to the egg and maintain a closed envelope around the developing embryo (Figs.

1–5). Cleavage of the egg occurs within this enveloping yolk-cell mass. The yolk cells follow or push into the cleavage furrows, apparently causing the wide separation of the blastomeres. In the late cleavage stages of the embryo of *Hydrolimax* a pattern of cell distribution emerges. The blastomeres, at first scattered among the yolk cells, aggregate toward the center of the yolk-cell mass, with the smaller blastomeres oriented toward the center of the embryo (Fig. 1). Cleavage continues until the embryo is a solid mass of cells (Fig. 2). In embryos slightly older than that of Figure 2 the mass of blastomeres, the main embryonic mass, is bilobed.

The cytoplasm of the blastomeres in the center of the embryo becomes less basophilic than the cytoplasm of surrounding blastomeres. A cavity appears among these lightly-staining cells and expands (Fig. 3). The cells which line the expanding cavity are derived from the lightly-staining blastomeres. *These cells lining the cavity are the epidermal (ectodermal) cells of Hydrolimax.* At stages of development slightly later than that of Figure 4, the cells lining the cavity are, at the light microscope level, similar in many respects to the epidermal cells of the adult *Hydrolimax.* It is this similarity which first drew my attention to the origin of the epidermis of this turbellarian. Because the cavity is lined by epidermis, it will be called the *epidermal cavity.* The epidermal cells flatten and spread as the cavity continues to expand. They increase in number, apparently through recruitment from the main embryonic mass, as no mitoses are seen among the epidermal cells.

The embryo approaches the surface of the enveloping yolk cell mass (Fig. 4). At this stage the embryo may be described as a mass of cells to one side of which is the expanding epidermal cavity. In Figure 4 the epidermal cavity is close to the surface of the yolk-cell mass and the main embryonic mass is centrally disposed. That portion of the embryo near the surface of the yolk-cell mass consists of a single layer of epidermis with a few associated embryonic cells. The covering yolk cells withdraw or are pushed aside by the expanding epidermal cavity. The epidermis thins and becomes perforated; the opening which appears unites the epidermal cavity with the environment within the cocoon. The epidermal cavity becomes obliterated by compression of the embryo by other embryos and yolk-cell masses within the cocoon (Fig. 5).

The epidermal cells spread out from the opening of the collapsed epidermal cavity as the embryo rises out of the yolk-cell mass. The embryo is thus completely inverted and comes to lie to one side of the yolk-cell mass (Fig. 6). During inversion and afterward, the epidermis, accompanied by a few internal embryonic cells, stretches posteriorly to cover and entrap the yolk-cell mass. Gastrulation in *Hydrolimax* is thus completed. At the close of gastrulation the embryo is bounded by a single layer of epidermal cells, beneath which are scattered the embryonic cells which accompanied the epidermis during epiboly. The anterior end of the embryo contains the main mass of embryonic (parenchymal) cells in which the brain (*cf.* Figs. 5 and 6) and pharynx are differentiating and from which will develop other definitive organs of the adult.

Discussion

The gastrulation of *Hydrolimax,* as described, is apparently unique. Compared with the gastrulation of other turbellarians, however, it parallels a two-part pattern of (1) differentiation of the epidermis and (2) movement of the epidermis to its definitive location (*cf.* Ball, 1916). The differentiation of the epidermis of *H. grisea* is manifested by a loss of basophilia by the ectodermal cells in the center of the embryonic mass and by the formation of the epidermal cavity among the epidermal cells.

Unique to *H. grisea* is the differentiation of the epidermal cells in the center of the embryo. In *Paravortex* (Ball, 1916) and in *Mesostoma* (Bresslau, 1904) the epidermal cells appear on one side of the embryonic mass, *after* the embryo has moved to one side of the yolk-cell mass. They flatten and spread, entrapping yolk cells and parenchymal cells. Bresslau's (1904) study of the embryonic development of *Plagiostomum girardi* revealed an interesting pattern of gastrulation as related to the early cleavage and distribution of blastomeres: (1) The micromeres are always directed toward the capsule wall of the cocoon. (2) The epidermis arises on the side of the embryo facing the capsule wall. The prospective epidermal cells migrate to the surface of the yolk-cell mass and differentiate, flattening and spreading. The central disposition of the small blastomeres of the embryo of *Hydrolimax* suggests a similar pattern: The small blastomeres are directed toward the center of the embryo. The epidermis arises in the center of the embryonic mass. However, studies of the lineage of the epidermal cells of *Hydrolimax* are incomplete.

In *Hydrolimax*, the formation and expansion of the epidermal cavity are the initial steps of gastrulation. The epidermal cavity is not a blastocoel (*cf.* Giesa, 1966). The expansion of the epidermal cavity apparently pushes the yolk cells aside and forces the epidermal cells to the outer surface of the yolk-cell mass where they can participate in the subsequent steps of gastrulation, inversion and epiboly. Inversion in the case of *Hydrolimax* is accomplished when the embryo, displaced from the center of the yolk-cell mass, opens out onto the surface of the yolk-cell mass. The inversion as described for *Hydrolimax* is not comparable to "inversion" as it occurs in certain sponge larvae: the flagellated choanocytes invert through the osculum of the larval sponge, forming an amphiblastula. The choanocytes assume their definitive position within the body of the sponge during gastrulation by a process of invagination (Okada, 1968).

The process of gastrulation of the embryo of *Hydrolimax* can perhaps be more appreciated and better understood by considering the environment in which the embryo develops: as a pre-meiotic oöcyte, and during meiosis, cleavage, and early gastrulation, the developing turbellarian is surrounded by yolk cells which contribute nothing directly to the definitive organs. The yolk cells provide nutriment to the embryo and to the juvenile turbellarian after it emerges from the cocoon. Each embryo in the cocoon is surrounded by its own yolk-cell mass. The yolk cells adhere closely to the egg. They push into the cleavage furrows and apparently cause or contribute to the initial scattering of the blastomeres through the yolk-cell mass. The blastomeres reaggregate within the center of the yolk-cell mass—by what process or force remains to be discovered. There the blastomeres organize and/or differentiate for the task of inversion, which is accomplished to a large extent by the expanding epidermal cavity.

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SUMMARY

1. Three events characterize the gastrulation of *Hydrolimax grisea*: formation of the epidermal cavity, inversion, and epiboly.

2. The epidermal cavity, the outstanding feature of gastrulation in this animal, forms in the center of the embryo among the lightly-staining cells of the future epidermis. The cavity expands, pushing aside the yolk cells which surround the embryo. Eventually the cavity becomes open at the surface of the yolk-cell mass. It is subsequently obliterated by compression from the other embryos within the cocoon.

3. Inversion begins when epidermal cells, which lined the epidermal cavity, spread around the yolk-cell mass from the opening of the collapsed cavity. The embryonic mass rises from the center to the surface of the yolk-cell mass, contributing to the progress of the inversion.

4. When the embryo is located to one side of the yolk-cell mass, epiboly begins. The epidermal cells stretch posteriorly to entrap the yolk-cell mass. A few parenchymal cells accompany the spreading epidermis.

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COELOMOCYTE AGGREGATION IN *CUCUMARIA FRONDOSA*: EFFECT OF ETHYLENEDIAMINETETRAACETATE, ADENOSINE, AND ADENOSINE NUCLEOTIDES

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Coelomocytes, a collective name for cells existing within the coelomic fluid of echinoderms, rapidly aggregate upon removal from the coelomic cavity. Descriptive accounts of the aggregation phenomena have been given by various workers (Bookout and Greenburgh, 1940; Boolootian and Giese, 1959; Endean, 1966), and a striking feature is the morphological change which occurs in one of the coelomocyte types, the bladder amoebocyte, and which appears to involve extensive membrane rearrangement.

Ethylenediaminetetraacetate (EDTA) was first used as a disaggregating agent by Anderson (1953) and is thought to act by chelating with divalent cations, in particular, calcium and magnesium, which, at the turn of the century, were shown to be of importance in cell aggregation (Roux, 1894).

Recently, much interest has been centered around the effects of adenosine and adenosine nucleotides on the aggregating behavior of cells; for example, adenosine diphosphate (ADP) has been shown to enhance platelet aggregation which can be reversed by adenosine or 2-chloroadenosine (Born and Cross, 1963), and adenosine triphosphate (ATP) has been found to inhibit the aggregation of embryo chick fibroblast cells (Knight, Jones, and Jones, 1966). Adenosine nucleotides have been implicated as playing an important physiological role in cellular adhesive and aggregating mechanisms which appear to reside at the cell surface (Jones, 1966).

In this study an attempt has been made to measure quantitatively the effects of EDTA, adenosine, and adenosine nucleotides upon coelomocyte aggregation in the holothurian, *Cucumaria frondosa*.

MATERIALS AND METHODS

Sea cucumbers weighing between 238 g and 425 g were obtained from Logy Bay, Newfoundland. They were kept in running sea water of temperature range 1° C to 3° C during the course of these experiments, which lasted from January to April. All needles and glassware coming into contact with coelomic fluid were siliconized with Siliclad (Clay-Adams). The substances to be tested for their effect upon coelomyocyte aggregation were used at the following concentrations: EDTA, 6×10^{-3} *M*; ATP, ADP, and adenosine monophosphate (AMP), all at 5×10^{-4} *M*. They were dissolved in an artificial sea water (ASW), calcium and magnesium free, of the following composition: sodium chloride, 28.326 g;

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sodium sulfate, 3.917 g; potassium chloride, 0.664 g; sodium bicaronate, 0.192 g; and potassium bromide, 0.096 g per liter of double-distilled water. Calcium- and magnesium-free sea water was used because EDTA and, to a lesser degree, ATP and ADP chelate with these divalent cations. If these compounds were dissolved in ordinary sea water, it would be difficult to assess what effective concentration of these compounds was coming into contact with coelomic fluid and coelomocytes.

Particluate matter was removed from the solutions by filtering through a millipore filter of 0.45 μ pore size. The pH of the solutions was adjusted to either pH 6.0 or pH 7.8 using 0.1 N HCl and 0.1 N NaOH, respectively, and was checked prior to each experiment. The pH of the resulting mixture of coelomic fluid and test solution was not measured.

Coelomcyte samples were obtained by piercing the dorsal body wall with a 22-gauge needle and withdrawing 0.1 ml of coelomic fluid into a disposable tuberculin syringe containing 0.1 ml of one of the test solutions. The two solutions were mixed by moving the plunger in the syringe gently to-and-fro several times. A drop of fluid was then placed in a chamber which was made as follows: two strips of Parafilm (Fisher), 1 cm apart, were placed on a microscope slide, and a glass coverslip was carefully lowered on top of the drop of fluid, with its edges resting upon the strips of Parafilm. The resulting chamber was sealed with Vaseline. At no time was the drop of fluid allowed to come into contact with either the Parafilm strips or Vaseline seal. The strips of Parafilm prevented the weight of the coverslip from causing damage to the cells.

Twenty minutes after the initial withdrawal of the coelomic fluid, the percentage of all cells that were not associated with a cell aggregate, that is, the free cells, was determined in the chamber. Of these free cells, the ratio, expressed as a percentage, of the number of bladder amoebocytes to the sum of bladder and filiform amoebocytes was enumerated in order to assess the tendency of the cells to undergo morphological change. The number of cell aggregates and the number of cells per aggregate were recorded as an indication of the intensity of the cellular aggregation process.

The chambers, solutions, needles, and syringes were kept at 4° C, except when the sample was withdrawn and the counting of the cells was done in the chambers. A minimum of 200 cells, both free and in aggregates, were counted. The same six animals were used for each solution at each pH value.

RESULTS

The effects of EDTA, ADP, and ATP upon coelomocyte aggregation are summarized in Table I. Of all the substances tested, only EDTA at pH 6.0 prevented both the morphological change from the bladder to filiform amoebocyte and the aggregation of coelomocytes. EDTA at pH 7.8 largely prevented cell aggregation but did not prevent the morphological change. Adenosine triphosphate at pH 7.8 appeared to enhance the morphological change of bladder to filiform amoebocyte. The results obtained with the remaining substances were not statistically significant from those obtained with ASW (Ca^{++} and Mg^{++} free) controls. The effect of pH alone upon coelomocyte aggregation was not found to be statistically significant when the data obtained for ASW (Ca^{++} and Mg^{++} free) at pH 6.0 and pH 7.8 were compared ($P > 0.05$).

TABLE I

Effects of EDTA, adenosine and adenosine nucleotides upon coelomocyte aggregation

	pH	Of all cells % free	Of free cells B/B + F × 100†
ASW*	6	65.82 ± 5.81	7.67 ± 2.27
	7.8	39.54 ± 9.72	4.98 ± 1.53
ASW*	6	89.70 ± 5.45**	93.87 ± 2.26***
+ EDTA 6 × 10^{-3} *M*	7.8	79.76 ± 7.39**	10.37 ± 2.04
ASW*	6	53.64 ± 9.50	7.98 ± 2.91
+ Adenosine 5 × 10^{-4} *M*	7.8	58.67 ± 7.82	1.83 ± 1.15
ASW*	6	54.25 ± 6.16	4.36 ± 3.09
+ AMP 5 × 10^{-4} *M*	7.8	52.88 ± 8.03	1.22 ± 0.88
ASW*	6	46.84 ± 11.24	6.72 ± 3.87
+ ADP 5 × 10^{-4} *M*	7.8	51.63 ± 9.04	0.68 ± 0.45
ASW*	6	56.18 ± 8.06	3.64 ± 2.71
+ ATP 5 × 10^{-4} *M*	7.8	47.31 ± 3.50	0.21 ± 0.20**

† Bladder amoebocyte/bladder + filiform amoebocyte × 100.
* ASW—Artificial sea water Ca^{++} Mg^{++} free.
** $0.01 > P > 0.001$ significant } With respect to ASW* at appropriate pH.
*** $P < 0.001$ highly significant }
Mean ± S.E.M. 6 animals.

The percentage of all the cells counted that were found in a given aggregate is shown in Figure 1. The intensity of the cell aggregation process was found to be minimal with EDTA at pH 6.0, and this finding is in keeping with the results described for EDTA at pH 6.0 upon the morphological change and cell aggregation. The very small number of aggregated cells obtained with EDTA at pH 6.0 were bladder amoebocytes, which were probably adjacent to one another purely by chance in the counting chamber and therefore were counted as a cell aggregate.

Coelomocyte morphology

It is relevant to this study to describe the more predominant coelomocyte types and to present the morphological changes which occur prior to and during cell aggregation. Figures 2–11 show these morphological changes as well as the major coelomocyte types.

The bladder amoebocyte (Figs. 2 and 3) is 15 μ–20 μ in diameter and possesses petaloid or bladder-like lobes which appear to undergo extensive morphological rearrangement to what is known as the filiform amoebocyte (Figs. 4 and 5) as a prerequisite to cell aggregation. Morula cells, appoximately 10 μ in diameter, are capable of movement and are characterized by several refractile granules within their cytoplasm when viewed with phase-contrast (Fig. 6). Figures 7 to 9 show motile cells which can exhibit a variety of different forms, and these have been called "lymphocytes" (Hetzel, 1963) because of their morphological and staining

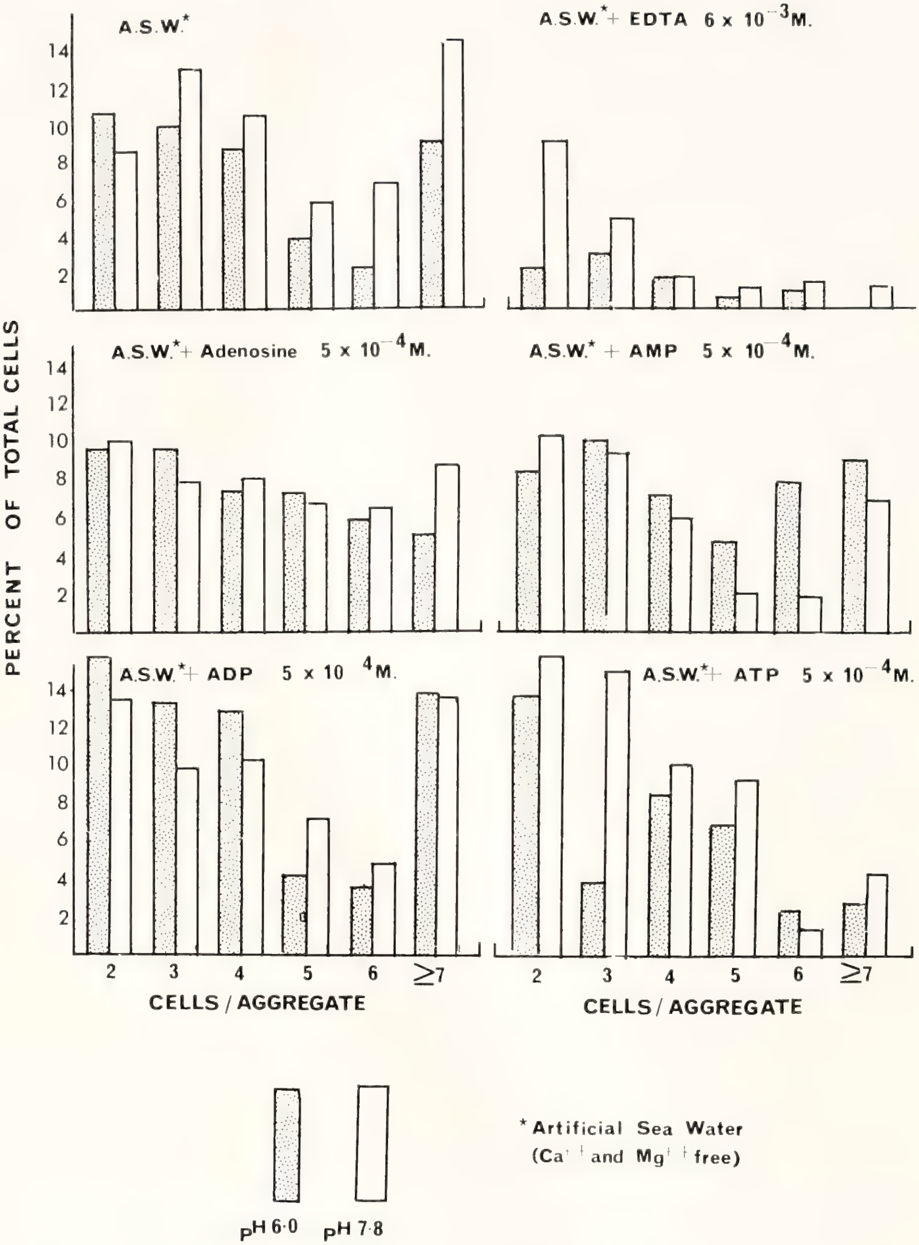

FIGURE 1. The percentage of total cells counted found in a given aggregate.

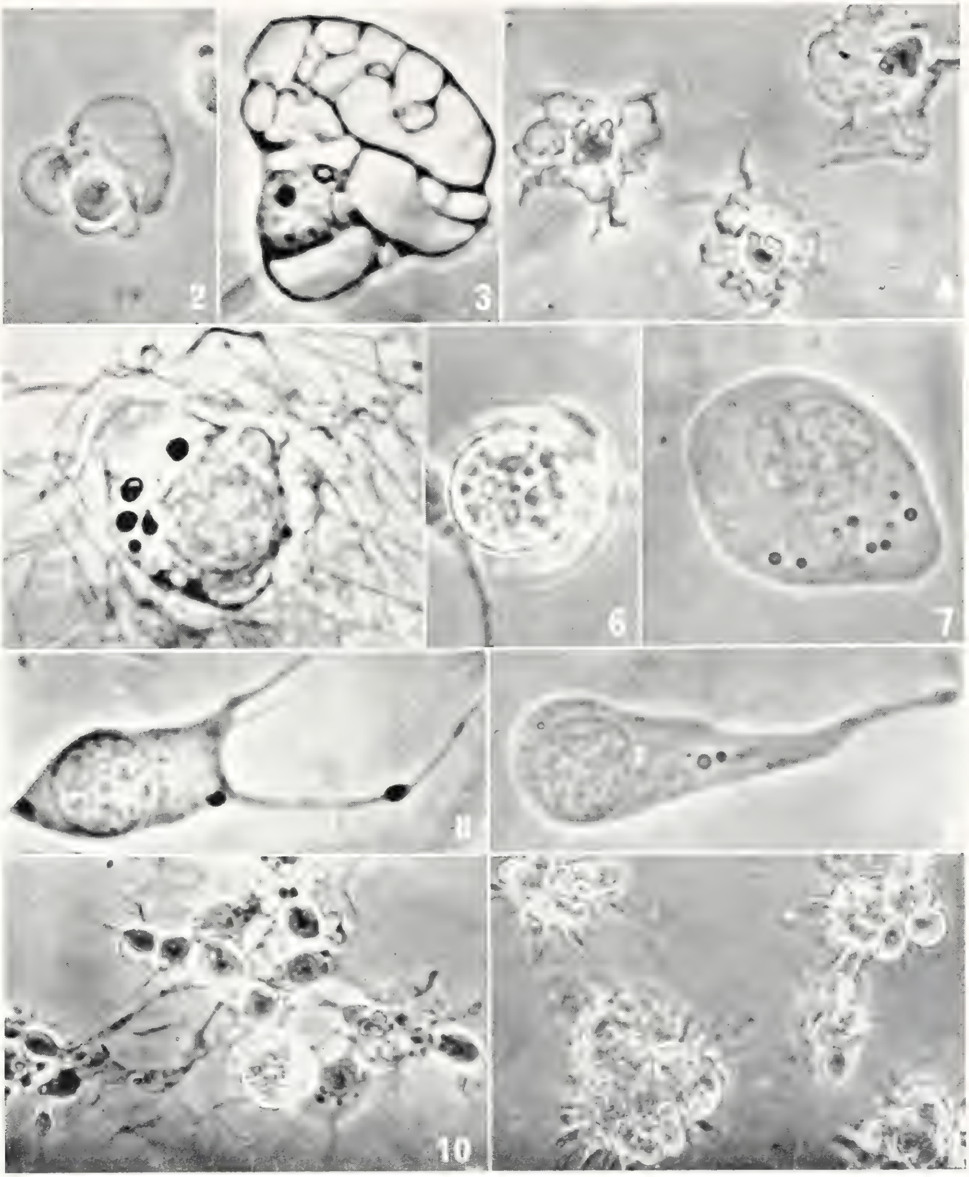

FIGURE 2. All photographs phase-contrast; Bladder amoebocyte, × 400. FIGURE 3. Bladder amoebocyte, × 1000. FIGURE 4. Filiform amoebocyte, × 400. FIGURE 5 Filiform amoebocyte, × 1000. FIGURE 6. Morula cell, × 1000. FIGURES 7, 8 and 9. Lymphocytes, × 1000. FIGURES 10 and 11. Typical coelomocyte aggregation, × 400.

similarities to mammalian lymphocytes. This is an unfortunate choice of terminology since it implies that these cells are analogous to mammalian lymphocytes which are known to be responsible for cell mediated and humoral immune mechanisms, properties which have not yet been shown to be possessed by echinoderm "lymphocytes". It is important to note that lymphocytes are notoriously deficient in morphological details, a fact contributing to the difficulties encountered in higher animals or associating the many physiological properties observed with this morphologically classified type of cell. Until such time that the known functions of echinoderm "lymphocytes" will suggest a functional terminology, a term should be used which denotes our ignorance regarding the functions of these cells and not one which implies properties as yet unproved.

Typical coelomocyte aggregates are shown in Figures 10 and 11.

Discussion

It has been shown that EDTA at pH 6.0 prevents both the morphological change and cell aggregation. At this pH, EDTA is reported to have a low stability coefficient for binding calcium ions, the greatest binding capacity for calcium being at pH 8.0 (Curtis, 1967). In this study, EDTA at pH 7.8 prevented aggregation but did not prevent the morphological change from bladder to filiform amoebocyte. These differences suggest that the cell aggregation phenomena can be divided into two different stages; one possibly requires calcium ions for the actual aggregation process, while the morphological change, and consequently cell aggregation, is prevented either by the removal of some other material which is chelated by EDTA at pH 6.0 or by EDTA reacting directly with the cell membrane or some other cellular component. In this regard it is pertinent to recall that L. Weiss (1960) pointed out that effective disaggregation of cells by EDTA is not proof that it does so by chelating with calcium ions.

Jones (1966) has recently put forward a unifying hypothesis of cell adhesion linking the adenosine nucleotides with contractile and relaxing properties of an actomyosin-like protein with ATPase activity located at the cell surface. For example, levels of ATP and ADP, by governing the physiological state of the contractile protein could, by initiating conformational changes in the membrane, bring about changes in the distribution of charges at the cell surface and hence alter adhesiveness and aggregating ability of cells. Evidence has been obtained using a variety of different cellular systems to support this concept. For example, glycerol-extracted cells of non-muscular origin, contracted in the presence of exogenous ATP (Hoffman-Berling, 1954), and contractile proteins have been isolated from many different cell types (Hoffman-Berling, 1956; Bettex-Galland, and Luscher, 1959; Loewy, 1952); ATPase activity has also been demonstrated in membranes of cells (Essner, Novikoff, and Masek, 1958; Novikoff, 1960).

No effect of adenosine or adenosine nucleotides upon coelomocyte aggregation was found in this study, with the possible exception of ATP at pH 7.8, which appeared to enhance the morphological change. One explanation of this latter result is that ATP provided an additional source of energy necessary for the membrane rearrangement which occurs in the bladder amoebocyte. An attempt was made to use a potentially more sensitive method of observing the effects of adenosine and adenosine nucleotides upon coelomocyte aggregation, that is, the turbidimetric method of Born and Cross (1963) which has been used extensively to follow aggregation of mammalian cells. However, in order to standardize the number of coelomocytes per cubic millimeter of fluid it was necessary to prevent aggregation by using EDTA at pH 6.0. Subsequent addition of adenosine and adenosine nucleotides did not produce aggregation as measured by a decrease in optical density and by direct microscopical observation. This finding is, of course, in keeping with the data presented in this study. The interpretation of this result is complicated, however, by the observation of Born and Cross (1963), who showed that platelets would not aggregate in response to ADP if the cells were in plasma which contained EDTA as an anticoagulant. One could argue that the presence of EDTA prevented any subtle effects of adenosine and adenosine nucleotides upon aggregation from being detected.

That adenosine and adenosine nucleotides, when used at concentrations which are effective in modifying avian and mammalian cell aggregation, fail to modify coelomocyte aggregation raises the question as to whether or not the mechanism of coelomocyte aggregation is similar to that postulated for other cell aggregating systems (Jones, 1966). To answer fully this question, studies are needed to see if contractile and relaxing proteins having ATPase activity can be isolated from coelomocyte membranes.

The result obtained with EDTA at pH 6.0 is also contrary to the known effects of EDTA at this pH upon mammalian and other cell aggregating systems. Many reports on the failure of EDTA to prevent cell aggregation and the enchancement of cell dispersion have been attributed to its poor binding capacity for calcium ions at this pH (Curtis, 1967). The mechanism by which EDTA at pH 6.0 stabilizes bladder amoebocyte membranes warrants further investigation.

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SUMMARY

Cells found within the coelomic cavity of the sea cucumber, *Cucumaria frondosa*, aggregated rapidly upon removal from the body. This cell aggregation is normally characterized by a morphological change in the bladder amoebocyte which becomes a filiform cell. A method has been devised whereby the effects of EDTA, adenosine, and adenosine nucleotides could be observed upon coelomocyte aggregation. It was found that EDTA at pH 6.0 in ASW (Ca^{++} and Mg^{++} free) prevented the morphological change from bladder to filiform amoebocyte as well as cell aggregation. EDTA at pH 7.8 in ASW (Ca^{++} and Mg^{++} free) did not prevent the morphological change although it did prevent cell aggregation. Based on the different chelating affinities of EDTA for calcium ions at these two different pH values, it is suggested that coelomocyte aggregation has two components: (1) the morphological change, which is Ca^{++} independent and possibly requires the removal of some other cation or is a direct effect of EDTA upon some

cellular component, and (2) the cell aggregation itself which appears to be Ca^{++} dependent.

Adenosine and adenosine nucleotides in general did not appear to influence coelomcyte aggregation. These results raise the question as to whether or not the mechanism of coelomocyte aggregation is similar to and can be described in the same terms as the mechanism currently used to explain avian and mammalian cell aggregation.

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LARVAL DEVELOPMENT OF *PAGURUS ANNULIPES* (STIMPSON, 1862) AND *PAGURUS POLLICARIS* SAY, 1817 REARED IN THE LABORATORY

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The hermit crabs *Pagurus annulipes* (Stimpson, 1862) and *P. pollicaris* Say, 1817 are common hermits found in shallow subtidal water from Massachusetts south to Florida. The southern distribution of *P. annulipes* reaches some distance north of Miami on the east coast of Florida and on the west coast of Florida extends from perhaps central or northwestern Florida westward along the perimeter of the Gulf of Mexico at least to Texas (Provenzano, 1959 and personal communication; Rouse, 1969). *P. pollicaris,* perhaps a subspecies, is also found along the northern perimeter of the Gulf of Mexico from western Florida to Texas (Provenzano, 1959). In the Woods Hole, Massachusetts region these two species and *P. longicarpus* Say comprise the shallow-water hermit crab fauna.

The ovigerous season of *P. annulipes* extends from about May to the end of September and completely overlaps that of *P. longicarpus.* The season of *P. pollicaris* is from early spring to the end of June. These seasons have been determined by examination of adults and of limited plankton samples during the summers of 1967 and 1968.

A review of the literature on larvae of members of the genus *Pagurus* has been given by Coffin (1960). All recent literature is cited by Roberts (1970) with the exception of Forss and Coffin, 1960; Kurata, 1964 and 1968; and Greenwood, 1966.

This paper deals with the external anatomy of *P. annulipes* and *P. pollicaris* larvae. Utilizing the recent description of *P. longicarpus* larvae (Roberts, 1970), two keys to the larvae of the shallow-water hermit crabs found in the Woods Hole, Massachusetts region are given, one based on larval pigmentation and the other on larval external anatomy. Comparisons are made with other species of the genus *Pagurus* in the context of examining current views on groups within the genus.

Materials and Methods

Ovigerous females were obtained from the Marine Biological Laboratory Supply Department of Woods Hole, Massachusetts during the summers of 1967 (*P. annulipes*) and 1968 (*P. pollicaris*). They were maintained in beakers until the eggs hatched. Larvae were transferred with a large bore pipette to small beakers and reared individually (*P. annulipes*) or in groups of 5 (*P. pollicaris*). Larvae were maintained at room temperature (24 ± 2° C) and at 16 ± 0.5° C (some *P. pollicaris*) in a controlled environment chamber with constant light. Water was changed and *Artemia* nauplii added as the culturing conditions dictated, usually daily or every other day. Larvae were examined daily to determine duration of each stage. Because no *P. pollicaris* successfully molted to the megalopa, duration for the fourth zoeal stage, was determined by assuming a successful molt. In addition to laboratory-reared specimens the verbal description of *P. annulipes* larvae utilized specimens obtained from the plankton collected just off the Bureau of Commercial Fisheries Laboratory, Woods Hole, Massachusetts.

Both fresh and preserved (70% ethanol) larvae were dissected with microneedles under the dissecting microscope. Details were checked using darkfield illumination. Verbal descriptions were based on 3 to 10 specimens except for carapace and total length. Depending on the stage, 4–108 specimens of *P. annulipes* (all planktonic) and 1 specimen of *P. pollicaris* were measured to determine lengths. Megalopal appendages of *P. pollicaris* were dissected out from incomplete molts, somewhat distorted and not fully expanded but adequate for description. Drawings were done from single specimens, using a camera lucida to give overall proportions with detail filled in by eye. Chromatophore patterns were determined from living larvae and larvae freshly killed in glycerine.

Throughout the descriptions the terminology of Roberts (1970) has been followed and the following abbreviations have been used: A1—antennule, A2—antenna, Mn—mandible, Mx 1—maxillule, Mx 2—maxilla, Mxp 1 to 3—first to third maxilliped, P 1 to 5—first to fifth pereiopod, Pl 2 to 5—second to fifth pleopod, U—uropod. The verbal descriptions have been abridged to report only variations of a given character among specimens, *i.e.,* information not illustrated in the figures.

RESULTS

As in other species of the genus, *Pagurus annulipes* and *P. pollicaris* have 4 zoeal stages and a megalopa. No variation in the number of stages was observed and no living prezoeae were observed.

Pagurus annulipes (*see Figs. 1–4*): *Zoea I*

Duration 5.4 days ($N = 21$).

Carapace length 0.9–1.3 mm, av. 1.1 mm ($N = 108$).

Total length 1.9–2.4 mm, av. 2.2 ($N = 108$).

Carapace without processes except for posterolateral spines. Rostrum unornamented, long, drawn out to a thin point, curved slightly ventrally, slightly longer than the antennae, which are longer than the antennules. Eyes sessile. Six abdominal segments, the sixth fused to the telson. Segments 2 through 5 with 2 pairs of posterodorsal and 1 pair of ventrolateral small spines, the ventrolateral spines of segment 5 only slightly longer than the others. Pleopod buds and uropods absent.

Red chromatophores predominate. One complex present around mouthparts extending from the base of the antennules posteriorly on each side of the labrum to the base of the mandibles. A single large red chromatophore on midlateral portion of each side of carapace; a large red chromatophore in abdominal segments 2 and 3; and a complex in segments 5 and 6-telson. One small medial yellow

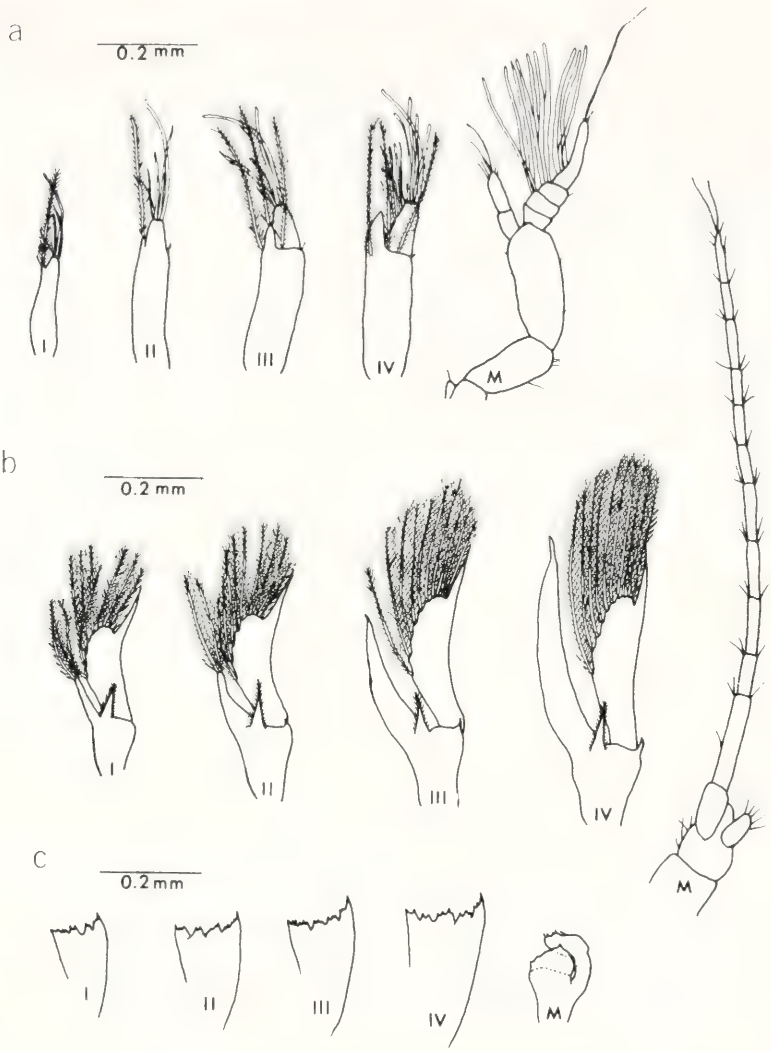

FIGURE 1. *Pagurus annulipes;* a. antennules, b. antennae, c. mandibles of zoeal stages I–IV and megalopa.

chromatophore in abdominal segment 6-telson. One large bar-shaped yellow chromatophore in cardiac region of thorax. Diffuse yellow pigment over dark pigment of eye.

A 1—With 1 long, 2 medium and 1 to 3 short terminal aesthetascs (A 1 similar in zoea II).

A 2—Scale with 8 plumose setae, 1 or 2 small setae medially.

Mx 1—Basal endite produced into 2 strong spines with 1 to 3 cuneate spinules each.

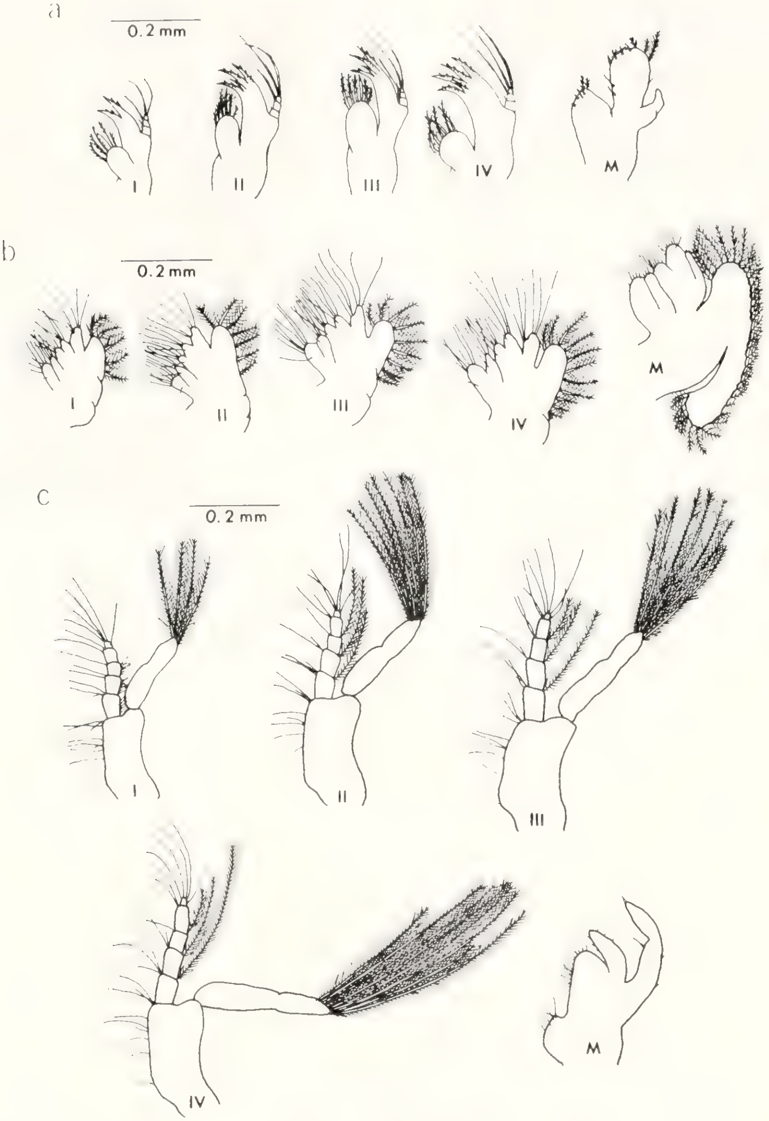
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FIGURE 2. *Pagurus annulipes;* a. maxillules, b. maxillae, c. first maxillipeds of zoeal stages I–IV and megalopa.

Mx 2—Distal lobe of coxal endite with 3 or 4 terminal setae (similar in zoea II, III, IV). Endopod unsegmented with 2 or 3 subterminal setae.

Mxp 1—Basis with 5 to 6 long and 2 or 3 short medial setae (similar in zoea II, III, IV).

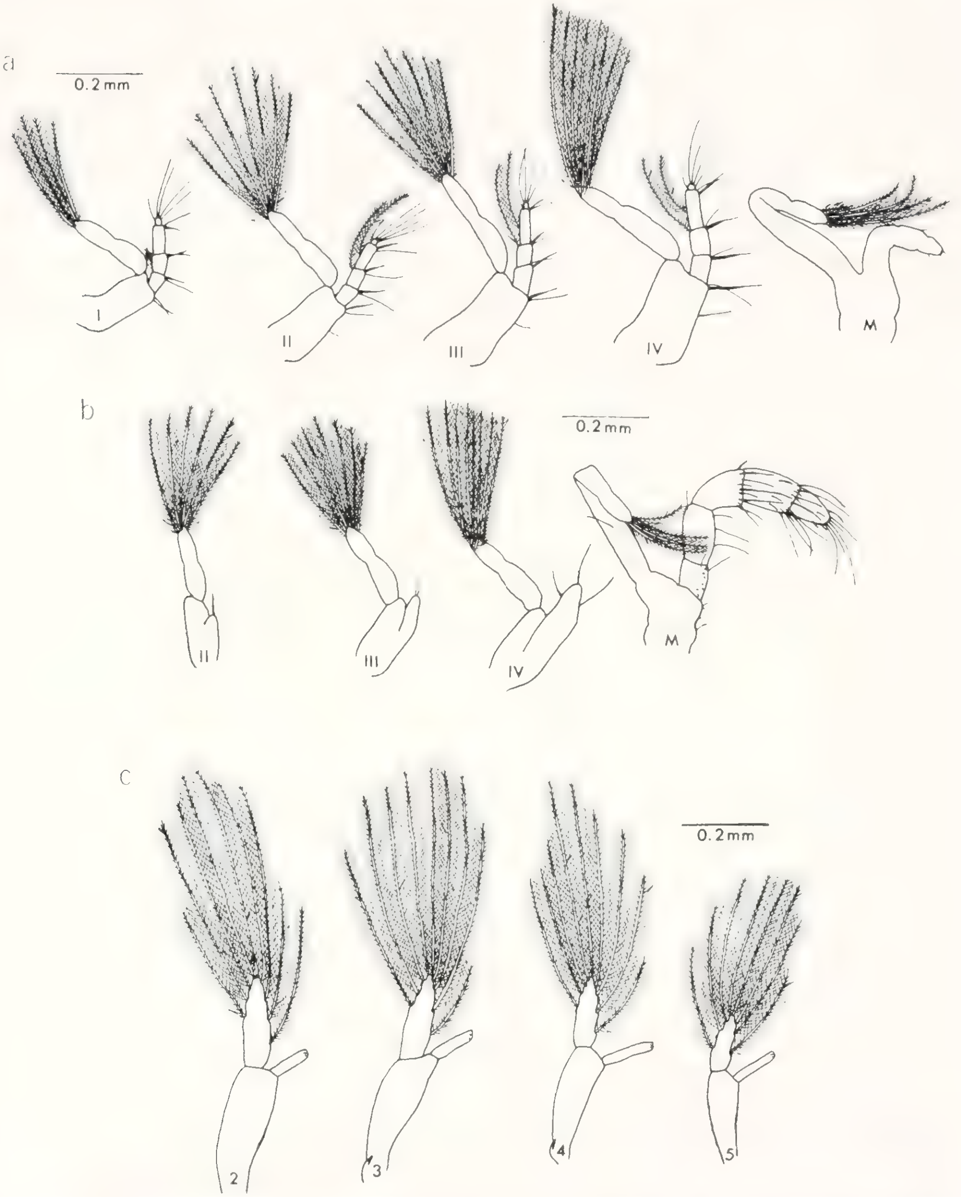
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FIGURE 3. *Pagurus annulipes;* a. second maxillipeds of zoeal stages I–IV and megalopa, b. third maxillipeds of zoeal stages II–IV and megalopa, c. second through fifth pleopods of megalopa.

Zoea II

Duration 3.4 days ($N = 14$).
Carapace length 1.3–1.7 mm, av. 1.5 mm ($N = 40$).
Total length 2.3–3.1 mm, av. 2.9 ($N = 40$).

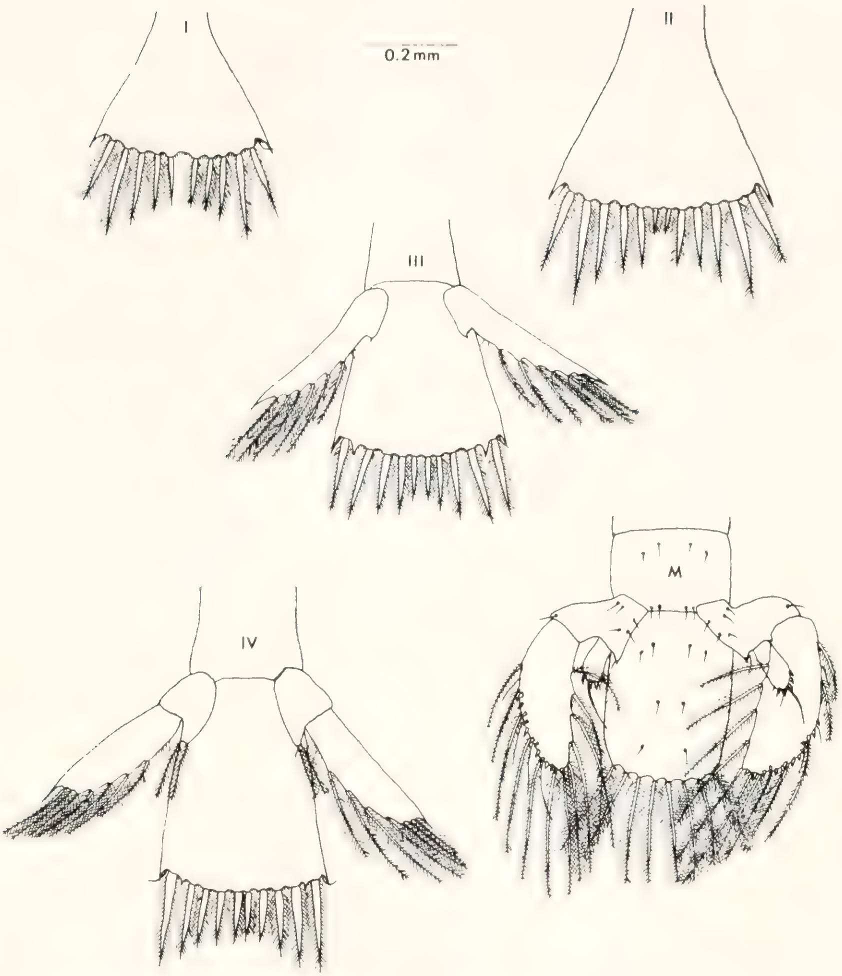

FIGURE 4. *Pagurus annulipes;* telsons of zoeal stages I–IV and megalopa, with uropods of zoeal stages III and IV and megalopa.

Mx 1—Basal endite with 4 strong spines, the 3 largest each bearing 2 or 3 cuneate spinules (similar in zoea III).

Mxp 3—Endopod bud with 1 or 2 terminal setae.

Zoea III

Duration 4.0 days ($N = 7$).

Carapace length 1.5–1.8 mm, av. 1.7 mm ($N = 10$).

Total length 2.8–3.2 mm, av. 3.0 mm ($N = 10$).

Sixth abdominal segment distinct, with uropods.

Pigmentation unchanged except for the appearance of small yellow chromatophores, 1 at the base of each eye and 1 medially in abdominal segment 2.

A 1—Outer ramus with 1 long and 2 or 3 shorter terminal aesthetascs plus 2 or 3 short setae. Distal end of peduncle with 1 to 3 small setae.

Zoea IV

Duration 2 days ($N = 1$).
Carapace length 1.9–2.1 mm ($N = 3$).
Total length 3.7–3.9 mm ($N = 3$).

Carapace, rostrum and abdomen segment number unchanged. Pleopod buds present.

Pigmentation unchanged except for the appearance of a yellow chromatophore at the base of each antennule.

A 1—Outer ramus with 1 long and 3 or 4 shorter terminal aesthetascs and 1 or 2 smaller setae also with 4 or 5 median aesthetascs. Distal end of peduncle with 2 or 3 small setae.

Mx 1—Coxal endite with 6 or 7 stout setae. Endopod with 2 or 3 terminal non-plumose setae.

Megalopa

Duration not determined.
Carapace length 1.3–1.7 mm ($N = 4$).
Total length 2.7–3.2 mm ($N = 4$).

Carapace nearly like adult. Posterolateral spines gone. Rostrum reduced and rounded. Eyes stalked with eye scales present. Abdomen with 6 segments, slightly asymmetric.

Pigmentation greatly elaborated. Of particular interest is incipient banding of the second and third pereiopods, suggesting the adult pattern.

A 1—Outer ramus terminated with 1 long and 2 or 3 short setae. Inner ramus with 5 or 6 short terminal setae.

A 2—Flagellum of 10 to 14 segments.

Mx 2—Proximal lobe of coxal endite with 5 or 6 setae. Proximal lobe of basal endite with 7 to 10 setae, distal lobe with 7 to 11 terminal and 2 subterminal setae. Scaphognathite with 35 or 36 plumose setae.

Mxp 1—Coxal endite with 4 or 5 setae.

P 4—Subchelate, propodus with a single row of 5 to 7 tubercles representing adult rasp, dactylus with 1 to 3 tubercles.

Pl 2 to 4—Exopod with 8 long and 1 (2) short plumose seta.

U—Exopod with 12 or 13 plumose and 3 to 5 shorter non-plumose setae and with 11 to 13 tubercles. Endopod with 2 or 3 non-plumose setae and with 4 or 5 tubercles.

Pagurus pollicaris (*see Figs. 5–10*): *Zoea I*

Duration at room temperature 3.8 days ($N = 26$), at 16° C 11.6 ($N = 17$).
Carapace length 1.6 mm, total length 2.8 mm.

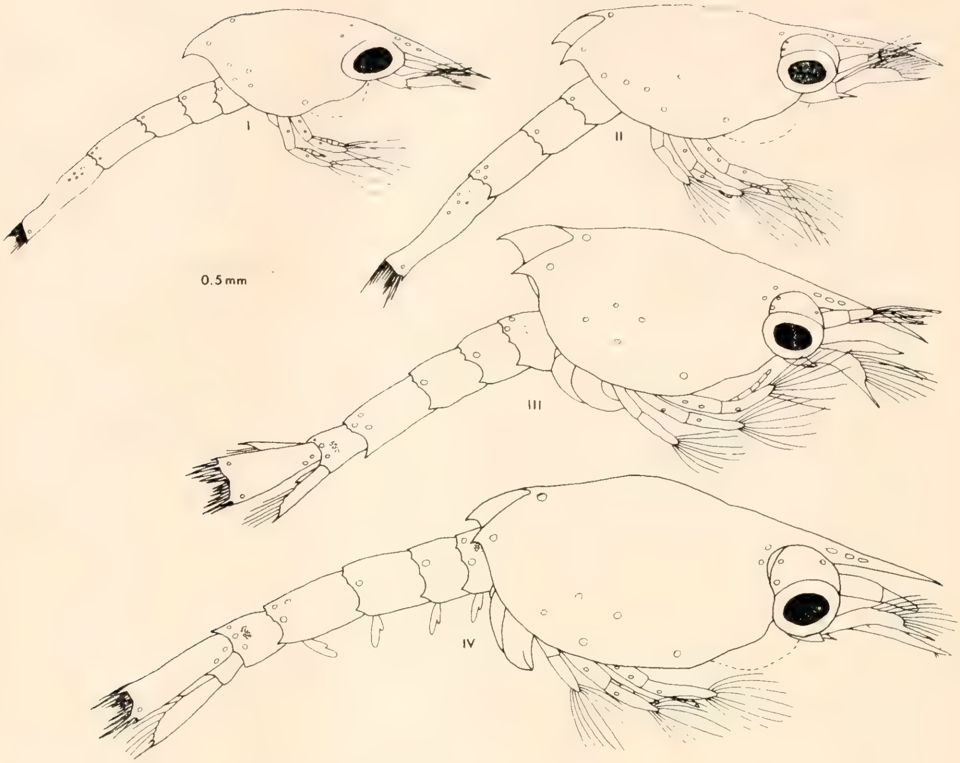

Figure 5. *Pagurus pollicaris;* zoeae I–IV, lateral view. Open circles represent yellow chromatophores; stippled, red.

Yellow chromatophores predominate. Three small yellow chromatophores on posterior half of rostrum. Red chromatophore complex from the base of the antennule posteriorly on each side of the labrum to the base of the mandibles. Yellow chromatophore towards base of each antennae. Two yellow chromatophores towards the outer margin of the bases and 1 towards the inner margin of the exopods of the first maxillipeds. One yellow chromatophore towards outer margin of the bases and 1 towards the inner margin of the exopods of the second maxillipeds. Carapace with 1 or 2 pairs of yellow chromatophores between the eyes, with single yellow chromatophore on midlateral edge of each side, with group of 4 to 6 yellow chromatophores towards posterolateral edge on each side, with 1 yellow chromatophore towards each posterolateral spine, and with a single yellow chromatophore at posteromedial edge. Abdominal segment 1 with 1 or 2 dorsal posteromedial yellow chromatophores, segment 2 with single or pair of posteromedial and a pair of posterolateral yellow chromatophores, segments 3 and 4 with pair of posterolateral yellow chromatophores, segment 5 with 3 pairs of posterolateral yellow chromatophores, segment 6-telson with pair of anteromedial red chromatophores followed posteriorly by 4 pairs of yellow

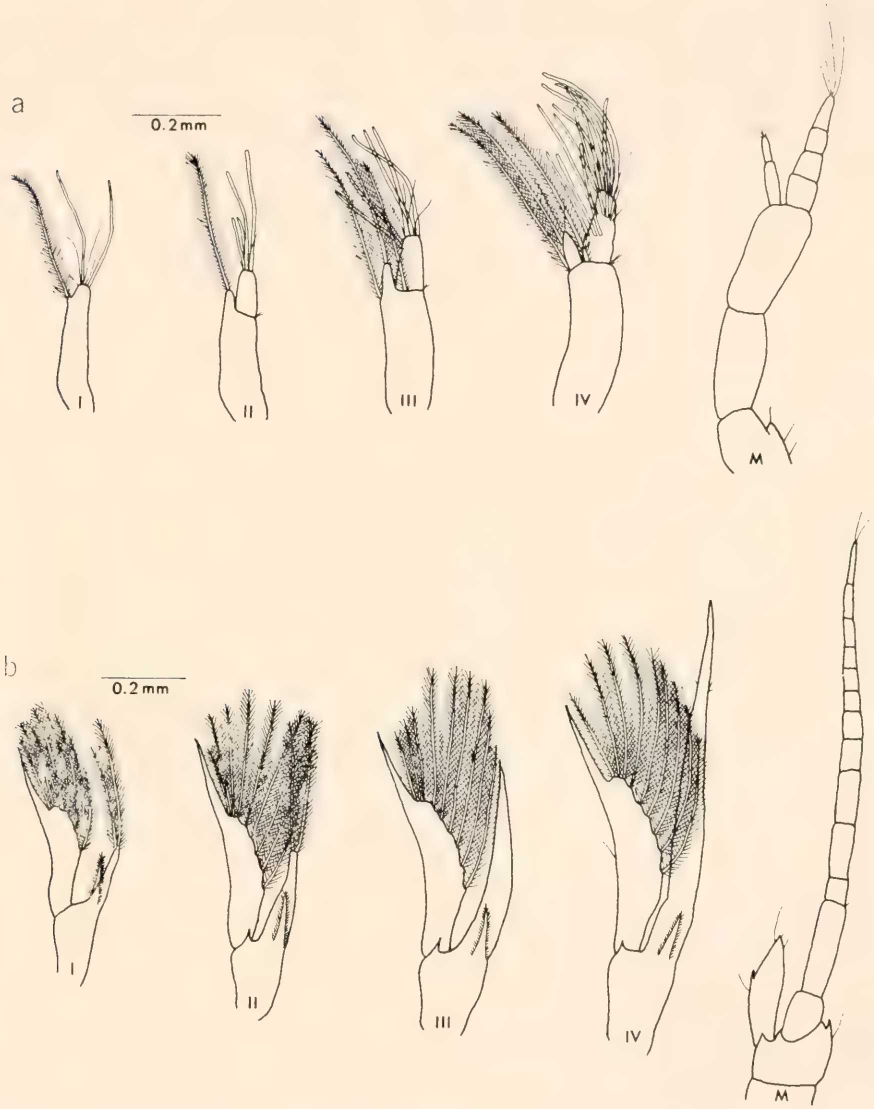

FIGURE 6. *Pagurus pollicaris;* a. antennules, b. antennae of zoeal stages I–IV and megalopa.

chromatophores plus a pair of posterolateral yellow chromatophores. Diffuse yellow pigment over dark pigment of eyes.

A 1—3 or 4 terminal setae.

Mx 1—Basal endite produced into 2 strong spines with 2 or 3 cuneate spinnules each.

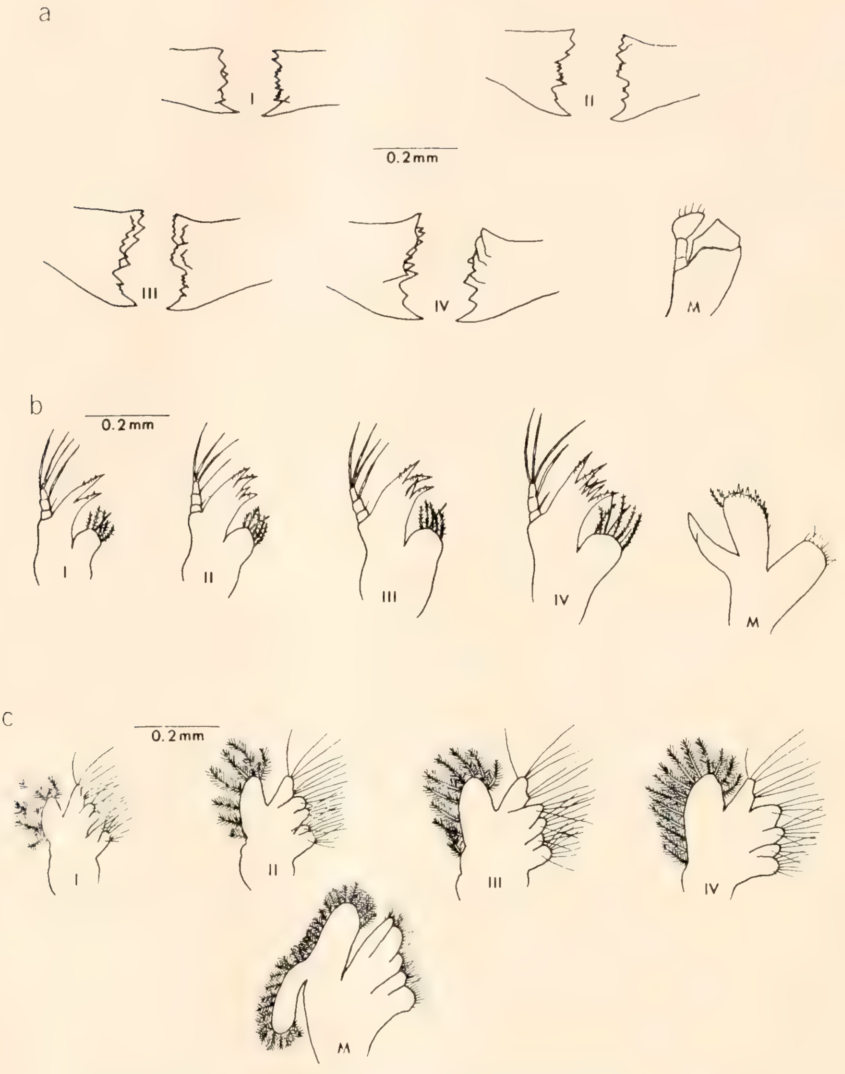
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FIGURE 7. *Pagurus pollicaris;* a. mandibles, b. maxillules, c. maxillae of zoeal stages I–IV and megalopa.

Mxp 1—Basis with 6 long and 3 or 4 short medial setae (Mxp 1 similar in zoea II, III, IV). Endopod segment 1 with 3–4 setae.

Mxp 2—Basis with 2 or 3 short medial setae (similar in zoea II, III, IV). Endopod segment 1 with 1 stout and 1 or 2 finer setae.

Zoea II

Duration at room temperature 3.2 days ($N = 46$), at 16° C 10.8 ($N = 11$). Carapace length 1.8 mm, total length 3.2 mm.

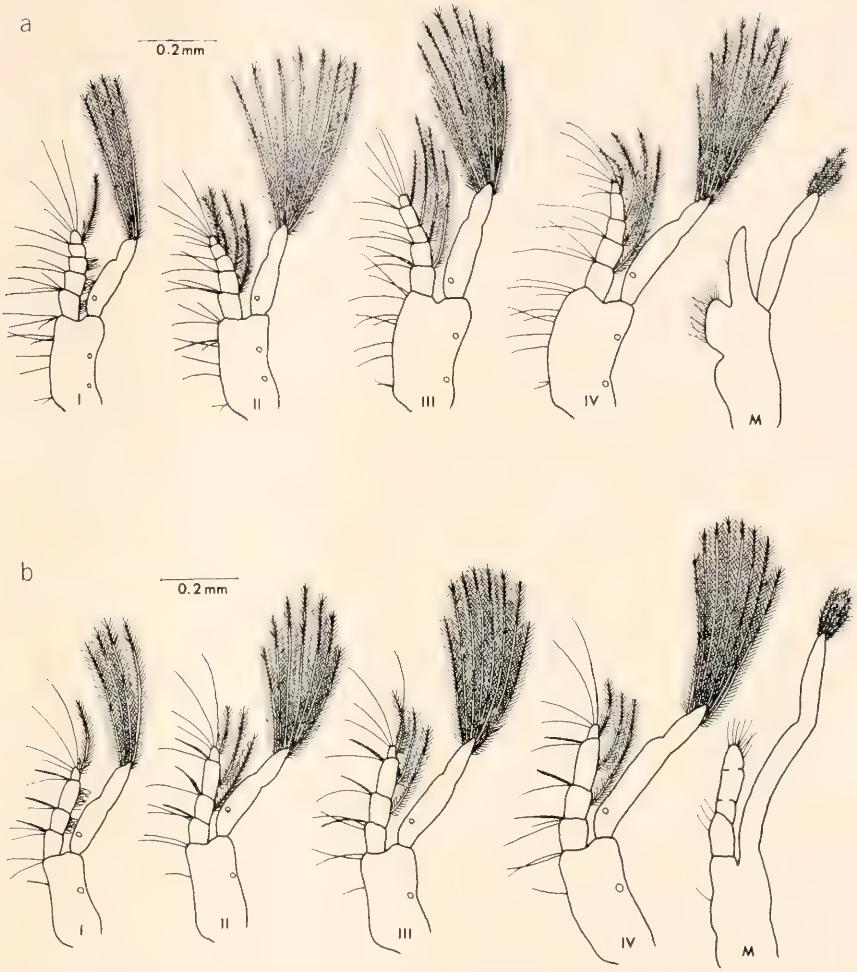

FIGURE 8. *Pagurus pollicaris;* a. first maxillipeds, b. second maxilipeds of zoeal stages I–IV and megalopa.

Pigmentation unchanged except for the addition of 1 or 2 posterior yellow chromatophores in each eye and of a median dorsal red bar-shaped chromatophore in abdominal segment 2.

A 1—Outer ramus with 2 or 3 short setae. Distal end of peduncle with 1 or 2 small setae on outer edge (similar in Zoea III).

Mx 1—Basal endite with 4 strong spines bearing 0 to 3 cuneate spinules (similar in Zoea III).

Zoea III

Duration at room temperature 3.6 days ($N = 33$), at 16° C 13.8 ($N = 9$). Carapace length 2.1 mm; total length 3.5 mm.

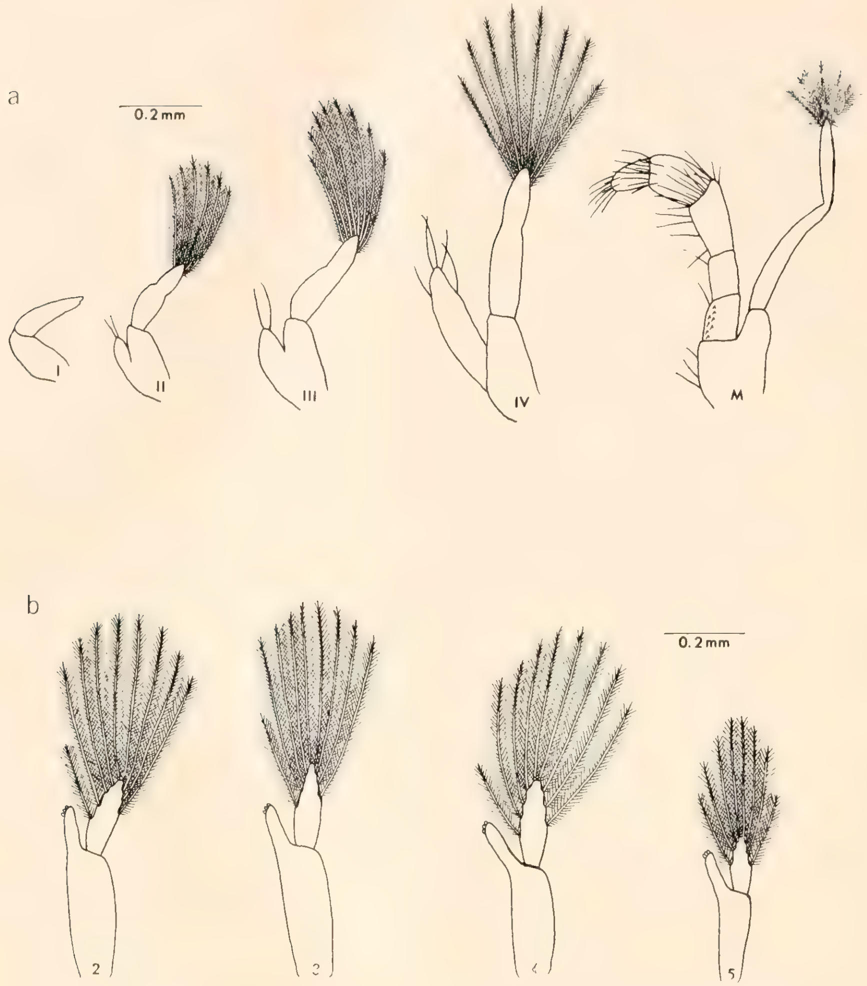

FIGURE 9. *Pagurus pollicaris;* a. third maxillipeds of zoeal stage I–IV and megalopa, b. second through fifth pleopods of megalopa.

Pigmentation unchanged except for the addition of 1 anterior yellow chromatophore in each eye.

A 1—Outer ramus with 3 or 4 setae.

Zoea IV

Duration at room temperature 4.6 days ($N = 15$), at 16° C 20.5 ($N = 2$). Carapace length 2.2 mm, total length 3.8 mm.

Pigmentation unchanged.

A 1—Outer ramus with 3 incipient segments; segment 3 with 5–6 terminal aesthetascs. Distal end of peduncle with 2 or 3 small setae.

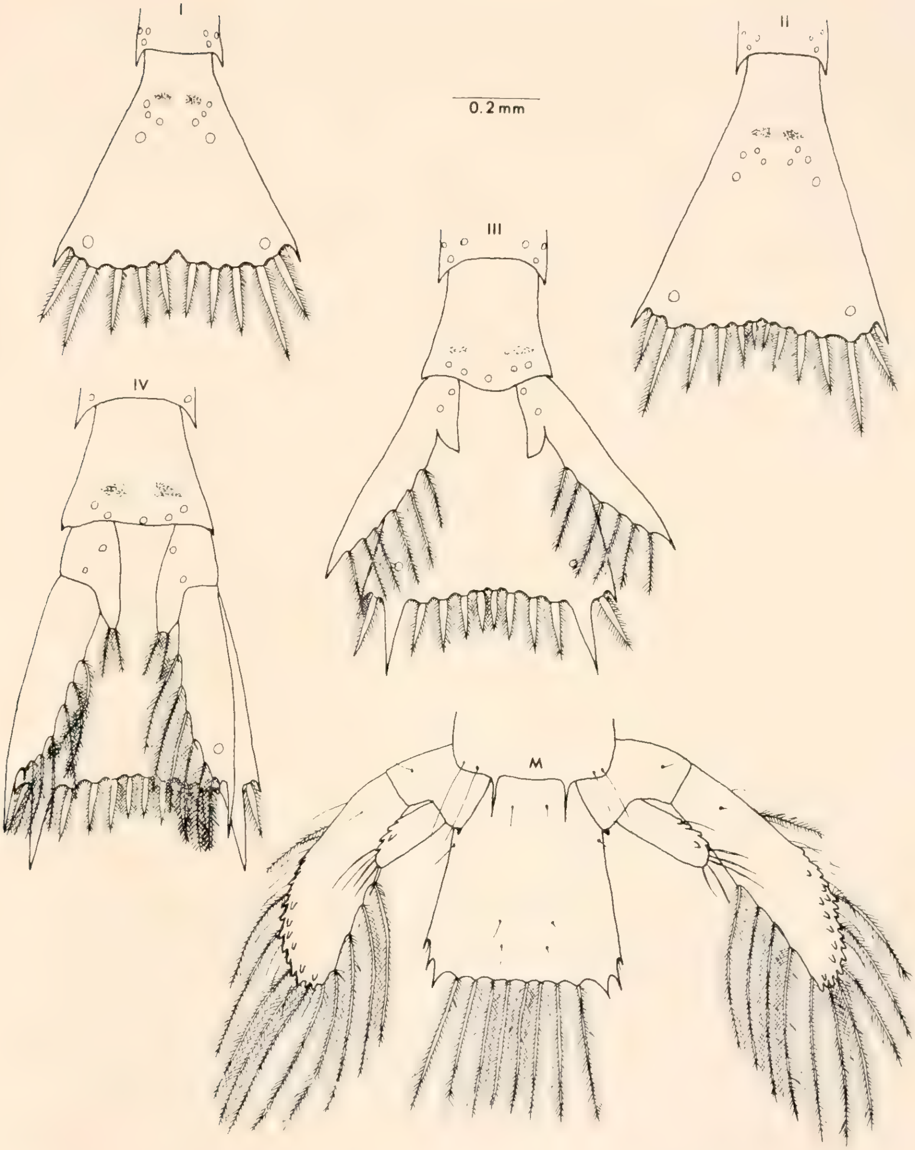

FIGURE 10. *Pagurus pollicaris;* telsons of zoeal stages I–IV and megalopa, with uropods of zoeal stages III and IV and megalopa.

Mx 1—Coxal endite with 7 or 8 stout setae.

Mx 2—Proximal lobe of basal endite with 5 or 6 terminal setae and 1 medial.

Megalopa

Duration, carapace and total lengths not determined.

Pigmentation not able to be determined, presumed to be predominantly yellow chromatophores. Aesthetasc number on A 1 not able to be determined.

A 2—Flagellum of 12 to 14 segments.

Mx 2—Proximal lobe of coxal endite with 8 to 10 terminal setae, distal lobe with 10 to 12. Proximal lobe of basal endite with 8 to 11 terminal setae, distal lobe with 8 to 12. Endopod unsegmented with 7 to 12 terminal setae.

Mxp 1—Exopod with 4 or 5 short plumose setae.

Mxp 2—Endopod with scattered short setae, 7 to 9 terminally. Exopod with 6 or 7 short terminal plumose setae.

Mxp 3—Exopod with 6 or 7 terminal plumose setae.

P 4—Subchelate, propodus with single row of 6 or 7 tubercles representing adult rasp, dactylus with 2 or 3 tubercles.

P 5—Propodus with tubercles in 4 rows totalling 14 to 16, dactylus with 3 or 4 tubercles and several short setae.

Pl 2 to 4—Exopod with 9(10) plumose setae.

U—Exopod with 10 or 11 long plumose setae and 8 to 12 shorter non-plumose setae, with 19 to 26 lateral and terminal tubercles. Endopod with 4 or 5 non-plumose setae, with 5 to 7 tubercles.

Larval key to three species of the genus Pagurus *found in the Woods Hole, Massachusetts region*

All species pass through 4 zoeal stages and 1 megalopa.

Key to Larval stages

1. Carapace without long rostrum, maxillipeds for feeding, 5 pairs of segmented functional pereiopods .... megalopa
   Carapace with long rostrum, maxillipeds with natatorial setae, pereiopods unsegmented or weakly segmented rudiments .... 2 (zoea)
2. Uropods present 3
   Uropods absent 4
3. Uropod with exopod free from basis .... fourth zoea
   Uropod unsegmented .... third zoea
4. Telson with 12 articulated plumose setae, third maxilliped functional with natatorial setae .... second zoea
   Telson with 10 articulated plumose setae, third maxilliped rudimentary without setae .... first zoea

Key to Species

For living or freshly killed larvae.

Zoeae and Megalopae

1. Few scattered mostly red chromatophores .... *P. longicarpus*
   Numerous chromatophores 2
2. Red chromatophores predominate: very large red chromatophore on each midlateral portion of carapace and large red chromatophore in abdominal segments 2–3, megalopal pereiopods 2 and 3 with incipient red banding ...... *P. annulipes*

Yellow chromatophores predominate: many on carapace at least 1 pair per abdominal segment, and one on posterolateral sides of telson ......*P. pollicaris*

For preserved larvae.

Zoeae

1. Ventrolateral spines of abdominal somite 5 long, almost reaching telson ....*P. longicarpus*
   Ventrolateral spines of abdominal somite 5 medium, not much larger than those of other somites 2
2. Maxillulary endopod with 3 terminal setae, telson process 4 of zoea III and IV longest ....*P. pollicaris*
   Maxillulary endopod with 2 terminal setae, telson process 4 of zoea III and IV very small ....*P. annulipes*

Megalopae

Telson formula 4 + 4: all processes long articulated plumose setae ....*P. annulipes*
Telson formula 5 + 5: process 1 minute spine, processes 2 to 5 long articulated plumose setae ....*P. longicarpus*
Telson formula 8 + 8: processes 1, 3, and 4 small spines, process 2 a fine hair, and processes 5 to 8 long articulated plumose setae ....*P. pollicaris*

DISCUSSION

In 1903 Thompson illustrated from plankton material the larval stages of what he considered to be *Pagurus longicarpus*, although he was aware of a possible confusion with *P. annulipes*. In comparing the preceding description of *P. annulipes* with Thompson's figures, the following differences are noted: third zoea slightly smaller than Thompson's; antennal scale setation of zoea I to IV 8 long and 1 to 2 short, 9 long and 1 short, 10 long, 10 long in this study compared with 9 long and 1 short for all four zoeal stages in Thompson's; maxillulary basal endite of zoea II with 4 spines compared with 3 spines in Thompson's; maxilliary scaphognathite setation of zoea II and III 7 and 9 compared with 6 and 10 in Thompson's; first and second maxilliped exopod setation of zoea II 7 compared with 6 in Thompson's; megalopal pleopod 2 to 5 setation 9 (10), 9 (10), 9 (10), 8 compared with 7, 7, 9, 7 in Thompson's. These differences are minor when compared with the overwhelming smilarity between his figures and this description. It is clear that Thompson in reality figured the larval stages of *P. annulipes*, not *P. longicarpus* (see Roberts, 1970). Although Thompson states that larvae of both species are found in plankton samples taken in Woods Hole during the summer, confusion arose from his assumption that, since the adults of *P. longicarpus* are considerably larger than those of *P. annulipes*, the larvae of *P. longicarpus* likewise would be larger. Just the opposite is the case; *P. annulipes* larvae are the larger.

Roberts (1970) has made a detailed comparison of *P. annulipes* and *P. longicarpus* in his Table II. In addition to the differences which he noted are the following: *P. annulipes* zoeae have a large bar-shaped yellow chromatophore in the cardiac region of the thorax, *P. annulipes* maxilliped exopod setation of zoea III is 7 compared with 8 in *P. longicarpus*, the uropod exopod of *P. annulipes* zoea IV has 1 terminal spur compared with 1 or 2 in *P. longicarpus*, and the megalopa of *P. annulipes* does not have a minute lateral spine on each side of the telson.

MacDonald, Pike and Williamson (1957) divide *Pagurus* larvae into two groups, A and B, separated by 9 zoeal and megalopal characteristics (see Roberts, 1970, Table III). Pike and Williamson (1960) propose a third group C, which shares 2 of the 9 characteristics with group A larvae, 5 with group B, and which has 3 similar to neither group. Roberts (1970) proposes a fourth group D for *P. longicarpus*, which shares 3 of the 9 characteristics with group A larvae, 4 with B, and in 2 is not similar to group A, B or C. *P. annulipes* larvae differ from those of group B in 3 respects: possessing a yellow chromatophore over the stomach region as in group A, having medium length lateral spines on abdominal segment 5 as in group C and having a megalopa with antennae about equal in length to the major cheliped as in group D. From the preceding description of *P. pollicaris* larvae it is seen that they differ from group B also in 3 respects: having telson process 4 of zoea III and IV long and fused with the telson as in group A, possessing medium length lateral spines on abdominal somite 5 as in group C and having a unique antennal scale setation.

Several points need to be made pertaining to the validity and value of present grouping within the genus *Pagurus*. Characteristics used to distingush the groups A, B, C and D are those observable without complete dissection of the larval appendages. Although not useful for quick identification, less easily observable characteristics of the appendages may be of equal or greater taxonomic significance. Another point is that complete larval descriptions for only 8 and partial descriptions for 9 *Pagurus* species have been reported (John C. Markham, School of Marine and Atmospheric Sciences, University of Florida, personal communication), out of more than 180 described species (Gordon, 1956). The 3 species most recently described would indicate the necessity of erecting a new group for each or of concluding that the presently proposed groups are neither taxonomically meaningful nor useful in facilitating plankton identification. The latter view is held here.

I am indebted to my wife, Beth, and Hank Roberts for technical help with the manuscript and figures and to Drs. David Egloff, Anthony Provenzano, Jr., Paul Illg, and Morris Roberts, Jr., for their critical reading of the manuscript. During the course of this study I was the recipient of a Public Health Service summer training grant, a Woods Hole Oceanographic Institution Summer Fellowship, a National Science Foundation Graduate Fellowship, and a Danforth Foundation Graduate Fellowship.

SUMMARY

1. The external anatomy of 4 zoeal stages and a megalopa of *Pagurus annulipes* and *P. pollicaris* is described from laboratory-reared specimens.

2. Keys to the shallow-water hermit crab larvae of the Woods Hole, Massachusetts region are given, based on larval pigmentation and on external anatomy.

3. Comparison with Thompson's (1903) figures shows that he illustrated *P. annulipes,* not *P. longicarpus.*

4. Comparisons with other larvae of the genus *Pagurus* are made in context of an examination of currently proposed groups within the genus. The validity and usefulness of these groups is seriously questioned.

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EFFECTS OF SUBZERO TEMPERATURES AND TRAWLING STRESS ON SERUM OSMOLALITY IN THE WINTER FLOUNDER *PSEUDOPLEURONECTES AMERICANUS*

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Since the freezing point of sea water (–1.8° C) is approximately one degree Centigrade lower than the freezing point of the serum of most marine teleosts (–0.7° C to –0.8° C), the possibility exists that these fish may encounter temperatures that would freeze their blood. Most arctic (Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957; Eliassen, Leivestad and Møller, 1960; Gordon, Amdur and Scholander, 1962; Leivestad, 1965), Antarctic (DeVries and Wohlschlag, 1969; R. N. Smith, personal communication) and temperate zone (Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957; Umminger, 1969a, b) fish avoid freezing at subzero temperatures by existing in a supercooled state. However, a few species of Antarctic fish of the genus *Trematomus* have been reported to lower the freezing point of their plasma to make it isosmotic with sea water (DeVries and Wohlschlag, 1969; Potts and Morris, 1968). Pearcy (1961) has also reported that the winter flounder, *Pseudopleuronectes americanus,* forms an "antifreeze" at subzero temperatures by lowering the freezing point of its serum. These data on supercooling and "antifreeze" formation in fish are summarized in Table III. Since *P. americanus* is the only marine teleost from the temperate zone that has been reported to form an "antifreeze' in winter, the present investigation was undertaken in an attempt to confirm these observations.

MATERIALS AND METHODS

The studies on temperature acclimation presented in this paper utilized laboratory-acclimated fish. Ten adult specimens of *P. americanus* were caught by trawl in Long Island Sound near Stonington, Connecticut, on January 3, 1967. Four of these fish were subsequently maintained in the laboratory on a photoperiod of eight hours of light per day at 15° C for five weeks; one fish was kept at 4° C for five weeks; three fish were kept at 4° C for four weeks and at –1.0° C for seven days; and one fish was maintained at 4° C for four weeks and at –1.5° C for seven days. The tenth fish was acclimated to 4° C for four weeks and transferred to water at –1° C; the temperature was gradually lowered to –1.8° C and the fish died. At autopsy, the tails of flounder lightly anesthetized with tricaine methanesulfonate (MS 222) were severed and the free-flowing blood was collected from the caudal artery. After centrifugation of the clotted blood, the serum was frozen at –20° C for later determinations of serum osmolality.

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TABLE I

Effect of temperature on serum osmolality of P. americanus

Temperature of acclimation	Fish no.	Sex	Serum osmolality (mOsm/liter)	Serum freezing point	Amount serum supercooled
15° C	1	M	373	−0.69° C	0° C
	2	M	423	−0.79° C	0° C
	3	M	403	−0.75° C	0° C
	4	M	355	−0.66° C	0° C
	Mean ± S.E.		389 ± 15	−0.72° C ± 0.02° C	
4° C	5	M	354	−0.66° C	0° C
−1.0° C	6	F	405	−0.75° C	0.25° C
	7	F	356	−0.66° C	0.34° C
	8	F	397	−0.73° C	0.27° C
	Mean ± S.E		386 ± 15	−0.71° C ± 0.02° C	0.29° C ± 0.02° C
−1.5° C	9	M	420	−0.78° C	0.72° C

Total osomolality, in reference to standard sodium chloride solutions, was estimated with a Mechrolab (new Hewlett-Packard) vapor-pressure osmometer equipped with Hamilton microsyringes. Statistical significance was determined using Student's *t* test.

In addition to the experiments on thermal acclimation, an investigation into the effects of trawling on serum osmolality was undertaken using ten freshly caught specimens of *P. americanus*. These fish were captured by trawl in water at 15° C from Long Island Sound near Old Saybrook, Connecticut, on October 14, 1966. The freshly caught fish were transported to the Essex Marine Laboratory in large steel wash buckets and autopsied at 15-minute intervals. Serum osmolality was determined (using the methods described above) for five fish killed 1–2 hours after capture and for five fish killed 5–6 hours after capture. There was no period of laboratory acclimation for these fish.

TABLE II

Effect of time after capture with trawl on serum osmolality of P. americanus

Group	Minutes after capture	Serum osmolality	Sex
Killed about 1–2 hours after capture	60	400	M
	75	392	M
	90	417	F
	105	412	F
	120	440	F
	Mean ± S.E.	412 ± 8	
Killed about 5–6 hours after capture	285	447	M
	300	448	M
	315	421	F
	330	531	F
	345	460	M
	Mean ± S.E.	461 ± 18*	

* Significantly different from group killed 1–2 hours after capture ($P < 0.05$).

TABLE III

Supercooling and "antifreeze" formation in marine teleosts

Family and species	Water temperature	Freezing point of serum	Amount serum supercooled	Reference
FAMILY ANARHICHADIDAE				
Anarhichas minor	−1.5° C	−0.80° C	0.70° C	Eliassen, Leivestad and Møller, 1960
FAMILY COTTIDAE				
Cottus scorpius	−1.5° C	−0.86° C	0.64° C	Eliassen, Leivestad and Møller, 1960
Gymnacanthus tricuspis	−1.73° C	−0.93° C	0.80° C	Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957
Icelus spatula	−1.73° C	−0.96° C	0.77° C	Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957
Myoxocephalus scorpius	−1.73° C	−1.44° C	0.29° C	Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957
	−1.7° C	−1.25° C	0.45° C	Gordon, Amdur and Scholander, 1962
FAMILY CYCLOPTERIDAE				
Cyclopterus lumpus	−1.5° C	−0.88° C	0.62° C	Eliassen, Leivestad and Møller, 1960
Liparis koefoedi	−1.73° C	−0.91° C	0.82° C	Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957
Liparis sp.	−1.9° C	−0.92° C	0.98° C	DeVries, 1970
FAMILY CYPRINODONTIDAE				
Fundulus heteroclitus	−1.5° C	−0.80° C	0.70° C	Umminger, 1969a
FAMILY GADIDAE				
Boreogadus saida	−1.73° C	−1.02° C	0.71° C	Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957
Gadus morhua	−1.5° C	−0.80° C	0.70° C	Eliassen, Leivestad and Møller, 1960
	−1.4° C	−0.76° C	0.64° C	Leivestad, 1965
Gadus ogac	−1.73° C	−1.47° C	0.26° C	Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957
	−1.7° C	−0.94° C	0.76° C	Gordon, Amdur and Scholander, 1962
Microgadus tomcod	−1.5° C	−0.98° C	0.52° C	Gordon, Amdur and Scholander, 1962
FAMILY NOTOTHENIIDAE				
Notothenia larseni	−1.87° C	−1.51° C	0.36° C	DeVries and Wohlschlag, 1969
Notothenia neglecta	−1.9° C	−1.08° C	0.82° C	Smith, 1968

TABLE III.—(*Continued*)

Family and species	Water temperature	Freezing point of serum	Amount serum supercooled	Reference
Notothenia rossii	−1.9° C	−1.07° C	0.83° C	Smith, 1968
Trematomus bernacchii	−1.73° C	−1.95° C	0° C*	Potts and Morris, 1968
	−1.9° C	−1.98° C	0°C*	DeVries, 1970
Trematomus borchgrevinki	−1.9° C	−2.07° C	0° C*	DeVries, 1970
Trematomus hansoni	−1.9° C	−2.01° C	0° C*	DeVries, 1970
Trematomus loennbergi	−1.9° C	−1.83° C	0.07° C	De Vries, 1970
Trematomus newnesi	−1.8° C	−1.01° C	0.79° C	Smith, 1968
FAMILY PLEURONECTIDAE				
Drepanopsetta platessoides	−1.5° C	−0.93° C	0.57° C	Eliassen, Leivestad and Møller, 1960
Pseudopleuronectes americanus	−0.80° C	−1.15° C	0° C*	Pearcy, 1961
	−1.0° C	−0.71° C	0.29° C	present paper
	−1.5° C	−0.78° C	0.72° C	present paper
FAMILY ZOARCIDAE				
Lycodes turneri	−1.73° C	−0.97° C	0.76° C	Scholander, van Dam, Kanwisher, Hammel and Gordon, 1957
Rhigophilia dearborni	−1.9° C	−1.52° C	0.38° C	DeVries, 1970

* Antifreeze present; freezing point of serum lower than temperature of water.

RESULTS

As indicated in Table I, the average serum osmolality of specimens of *P. americanus* acclimated to −1° C was not significantly different from the serum osmolality of fish acclimated to 15° C. The serum osmolality of flounder at −1° C was 388 mOsm/liter which corresponds to a serum freezing point of −0.71° C. These fish formed no "antifreeze," being supercooled by 0.29° C. Similarly, the serum osmolality of a single fish held at −1.5° C was 420 mOsm/liter; this fish was supercooled by 0.72° C. A single fish was placed into water at −1° C and the temperature was gradually lowered to −1.8° C over a seven-day period. At this temperature, ice began to form at the surface of the sea water and tiny crystals of ice began to circulate throughout the water in the aquarium. The presence of these crystals of ice caused the fish to freeze solid and die; presumably, the ice crystals seeded the supercooled blood, causing it to freeze.

Studies on freshly caught flounder (Table II) at 15° C showed that serum osmolality increased from 412 mOsm/liter in fish killed 1–2 hours after capture to 461 mOsm/liter in fish killed 5–6 hours after capture. Not only did serum osmolality increase significantly with time after capture, but also the serum osmolality of the freshly caught fish at 15° C (437 mOsm/liter) was significantly higher than the serum osmolality of fish acclimated to the laboratory at 15° C (389 mOsm/liter).

DISCUSSION

Pearcy (1961) found that specimens of *P. americanus* living in the Mystic River estuary in Connecticut during the winter of 1958–59 often encountered temperatures as low as –0.8° C. These winter fish had an average serum freezing point of –1.15° C. In contrast, the serum freezing point for fish collected in the summer was –0.63° C. In the present investigation, the serum freezing point (–0.71° C) of cold-acclimated winter fish is more similar to the summer than to the winter values reported by Pearcy (1961). What, then, accounts for the discrepancy in the data from these two studies?

The only obvious difference in the procedures used in the two investigations concerns the handling and capture of the fish. Pearcy (1962) used freshly caught flounder that may well have been stressed by the trawling, whereas the present investigation deals with fish that had been acclimated to the laboratory for several weeks. The present study shows that capture by trawl can elevate the serum osmolality of *P. americanus*. Therefore, the trawling procedures employed by Pearcy (1961) may account for the exceedingly high values he obtained for serum osmolality in winter fish. Similar studies by Slicher, Pickford and Pang (1966) on *Fundulus heteroclitus* showed that the serum osmolality was elevated in fish unaccustomed to handling when compared with fish "trained" to be familiar with the handling procedures. Therefore, Pearcy's account of "antifreeze" formation in the winter flounder at low temperatures was probably due to an increased serum osmolality produced by trawling stress during capture.

Another possible reason for the discrepancy in these two studies is that there may be yearly variations in the ability of the flounder to produce its "antifreeze." For example, Gordon, Amdur and Scholander (1962) were unable to find the high serum osmolalities reported by Scholander, van Dam, Kanwisher, Hammel and Gordon (1957) in two species of arctic fish. Gordon, Amdur and Scholander (1962) concluded that the difference in the serum osmolality measured during different years was real and that the amount of "antifreeze" added to the blood during the winter was extremely variable from year to year. This may well be the case with the winter flounder also.

Pearcy (1961) also found that winter flounder kept in the laboratory would freeze and die at temperatures between –1.0° C and –1.5° C. These lethally low temperatures were similar to the serum freezing points of freshly captured winter fish. Pearcy concluded that the flounder could not survive temperatures much below –1.15° C since this was the freezing point of the serum. Apparently, these data show that winter flounder cannot exist in a supercooled state. However, the present study shows that the winter flounder definitely can survive in a supercooled state. The fish freeze and die only when ice crystals are present in the water to nucleate the supercooled blood. In nature, surface ice is probably seldom encountered because winter flounder are bottom fish that often cover themselves with mud or sand. The ability of the winter flounder to avoid freezing by existing in a supercooled state (as reported in this paper) is the same mechanism utilized by the majority of cold-hardy marine teleosts (Table III) in surviving the subzero cold.

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SUMMARY

1. The average serum osmolality of winter flounder, *Pseudopleuronectes americanus,* acclimated in the laboratory to –1° C (386 mOsm/liter) was not significantly different from the serum osmolality of fish at 15° C (389 mOsm/liter).

2. Winter flounder survived temperatures as low as –1.5° C in a supercooled state. When ice crystals were present in the sea water at –1.8° C, the fish froze and died due to nucleation of the supercooled blood.

3. The serum osmolality of freshly caught flounder increased significantly with time after capture (up to six hours) and was significantly higher than the serum osmolality of laboratory acclimated fish at the same temperature.

4. A previous account of "antifreeze" formation in the winter flounder at low temperatures was probably due to an increased serum osmolality produced by trawling stress during capture.

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